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in ovo model

Melissa Russ
University of Wollongong

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**An investigation of the effects locust-control pesticides,
Fenitrothion and Fipronil, on avian development
using an *in ovo* model**

Melissa Russ, B.Sc.

**A dissertation submitted in partial fulfillment of the requirements for the degree of
Masters of Science at the University of Wollongong**

March 31, 2005

DECLARATION

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person where due reference is not made in the text.

ACKNOWLEDGEMENTS

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Abstract

Locust control operations, carried out by the Australian Plague Locust Commission (APLC), typically coincide with times of insect abundance as well as the breeding periods of many oviparous Australian vertebrates. Maternal exposure and storage of pesticides can result in the subsequent deposition of these lipophilic chemicals within the yolk lipids. Thus, these chemicals can be sequestered within an egg, exposing the embryo throughout its *in ovo* development. Fenitrothion and fipronil are pesticides currently in use by the APLC. A few teratogenic studies have been conducted regarding *in ovo* exposure to fenitrothion, though exposure occur during the later stages of development and was not modeled as maternal deposition in yolk. To date no *in ovo* study has investigated the effects of fipronil on development.

The lethal effect of fipronil varies widely between species, making toxicity to a given species difficult to predict. Limited research on rats has suggested that fipronil may adversely affect normal thyroid function, causing an increase in thyroid hormone clearance and related changes in thyroid hormone levels and regulation. Using these findings as a starting point, further investigation was warranted into the full extent of fipronil's interaction with the thyroid system.

Eggs of the domestic chicken were treated *in ovo* at day 3 of development with either fenitrothion or fipronil over a range of doses. Controls included groups that received the oil vehicle only or that received no injection. Measurements oxygen consumption of embryos were made on days 12, 14, 16, 17 and 18 of incubation. Upon

hatch, body mass and skeletal lengths of the skull and tarsus were measured. Blood was collected and analyzed for either cholinesterase inhibition (a biomarker for fenitrothion exposure) or plasma thyroxine levels (for fipronil treated eggs).

Embryos treated with fenitrothion did not differ significantly between treatments in hatchability, body mass or skeletal measurements. Plasma total, acetylcholinesterase and butyrylcholinesterase activities followed a weak linear pattern, increasing as the dose increased, but were not significantly different from controls. Oxygen consumption for the control group was significantly higher than the fenitrothion 0.1 and 5.0 mg/kg treatment groups, however none of the treatment groups differed significantly from the oil-injected group.

Embryos treated with fipronil in general exhibited lower hatchability at the highest doses, although there were no statistically significant differences between fipronil treated groups and the controls. There were also no statistically significant differences between treatments in body mass or skeletal measurements. While plasma thyroxine levels in general increased with increased dose, there were no statistically significant differences between the fipronil and the oil-treated groups. Oxygen consumption of the embryos in the control group was higher than fipronil-treated groups over time, but there were no statistically significant differences between dose treatments.

In a separate pilot study, I gave breeding female Zebra Finches oral doses of fipronil (100, 200 and 500 mg/kg and control oil) to determine the extent of maternal

transfer of the pesticide to the egg yolk. All eggs laid by these females were collected within a day of laying for 3 weeks after treatment and analysed for fipronil residues. Although the total number of eggs laid by treated females was small, there was a weak dose dependency in fipronil deposition in yolk. The degree to which this may affect embryo development was not examined as part of this thesis.

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Chapter 1

Review of the effects of Fenitrothion and Fipronil on non-target vertebrates

1.1 Introduction

In eastern Australia, the Australian Plague Locust Commission (APLC) is responsible for preventing agricultural losses due to locust infestations. To accomplish this, the APLC monitors locust activity and controls locust outbreaks when required by aerial application of insecticides. Typically, bands of flightless, juvenile locusts are identified and exterminated before they become flying adults that form voracious, swarms. To date, the APLC has used the organophosphate fenitrothion for most of their spray operations because it is highly effective as well as being the most cost-effective pesticide available to the Commission (Story and Cox 2001). In addition, fenitrothion rapidly breaks down in the environment (Roy et al. 1996) as well as *in vivo*, becoming almost completely metabolized with only very limited bioaccumulation (Escartin and Porte 1996; Tsuda et al. 1995). However, fenitrothion like other organophosphates (OP) is a cholinesterase inhibitor and, because these enzymes are present in vertebrates as well as invertebrates, fenitrothion can act as a biocide across species. Therefore, toxicological effects of exposure in non-target organisms are often observable and can be elicited at low doses (Holmes and Boag 1990).

The risk of fenitrothion having sub-lethal effects on non-target species has prompted the APLC to investigate and trial the use of a relatively new pesticide, the phenyl pyrazole fipronil. Fipronil acts as a γ -aminobutyric acid (GABA) inhibitor and

has higher toxicity at a given dose to invertebrate species than to vertebrates. In addition, when used for locust control, lower concentrations than fenitrothion are required to eradicate a locust population (Story 2001). However, data regarding non-target species toxicity, sub-lethal effects or teratogenicity are lacking. Information exists regarding the lethality of fipronil to target insects including LD50's (dose that is lethal to 50% of the population), rates of mortality, and percent mortality at various doses. However lethal doses for other organisms are only known for a handful of species, which includes most pest species, some invertebrates and a few avian species (Tingle et al. 2003). Clearly, more investigation of the sub-lethal effects of fipronil application on vertebrates is needed (Dinham 2000).

Endocrine-disrupting effects are important when describing chemical toxicity. If evidence exists that suggests that a contaminant interferes with hormonal systems, further investigation is critical to understanding the full impact of these pesticide applications on ecosystem health. Some evidence suggests that fipronil adversely affects normal thyroid function (Hurley, Hill and Whiting 1998), and further investigation is needed in order to fully understand fipronil's interaction with the thyroid system.

Locust outbreaks usually occur following a period of moderate rainfall. Adults are stimulated by soil moisture to lay their eggs and within two or three weeks, young locusts begin to hatch and aggregate. At the same time, rains can stimulate aggregations and breeding in numerous bird species in the same area due to the increase

in food abundance. Insectivorous birds, for example raptors and swallows, have been observed gorge feeding on locusts as well as other insect species that had been exposed to pesticides (Norelius and Lockwood 1999). Likewise, granivorous birds, such as parrots and finches, have been observed consuming seeds or grasses sprayed with pesticide (Astheimer, personal communication). Because of fipronil's lipophilic nature it can be incorporated into the fat stores of the animal ingesting it.

The previous toxicity studies regarding fipronil toxicity focus on the effects elicited in adult animals, but there has been very little investigation of developmental effects. Due to the coincidence of rainfall and increased insect abundance, spraying events in eastern Australia typically coincide with the breeding periods of native fauna, including avian species. Female birds with accumulated stores of fipronil may transfer pesticide into egg yolk during yolk deposition, making it available to their developing young. Thus fipronil sequestered in yolk has the potential to alter normal development and possibly permanently alter the fitness of the offspring.

In this chapter I review the properties of fenitrothion and fipronil and the literature pertaining to their effects on non-target organisms, particularly vertebrates, to provide background for the research I conducted.

1.2 Fenitrothion

1.2.1 Chemical Properties

Fenitrothion (0,0-dimethyl 0-4-nitro-*m*-tolyl phosphorothioate), while strongly stable in storage, (Miles et al. 1979) degrades quickly under both laboratory and field conditions into a multiplicity of metabolites (Figure 1.1; Greenhalgh, Dhawan and Weinberger 1980; Roy et al. 1996). The fenitrooxon form can be created during metabolism as well as after exposure to ultraviolet light (Birmelin et al. 1998; Greenhalgh and Marshall 1976), and can be even more toxic

than the parent Compound (Escartin and

Figure 1.1 Common degradation pathways and metabolites of fenitrothion (Greenhalgh et al. 1980)

Porte, 1996). Degradation in alkaline environments is accelerated and produces the 3-methyl-4-nitrophenol as the main metabolite (Greenhalgh, Dhawan and Weinberger 1980). This moiety is also formed by hydrolysis via organisms such as the fungus *Trichoderma viride* and has a greater mobility due to the fact that it is more water soluble than fenitrothion (Baarschers and Heitland 1986; Baarschers, Elvish and Ryan

1983). The demethylfenitrothion form is produced in acidic conditions (Greenhalgh, Dhawan and Weinberger 1980) and the aminofenitrothion form has been detected from the breakdown of fenitrothion by microbes such as bacteria and fungi (Adhya, Sudhakar-Barik and Sethunathan 1981). Photodegradation changes the position of the sulfur to produce the S-methyl entity (Durand et al. 1994; Fukushima et al. 2003).

1.2.2 In vivo Effects of Fenitrothion

The metabolism of fenitrothion is inconsistent and species dependent, perhaps due to variation in liver oxidation activities (Mihara, Misaki and Miyamoto 1979). Within insects such as the spruce budworm, between 43% and 54% of the different pesticide formulations remained on the cuticle, failing to even penetrate the organism (Sundaram 1988). Of the amount that was absorbed the main products generated were demethylfenitrothion and its degradation product nitroresol, which is also produced directly from fenitrothion degradation (Sundaram 1988). In birds, as well as the red swamp crayfish, *Procambarus clarkii*, the major metabolite is 3-methyl-4-nitrophenol, its sulfate conjugate and the fenitrooxon (Mihara, Misaki and Miyamoto 1979; Birmelin et al. 1998) whereas in triatomine reduviid bugs, *Triatoma infestans*, the main metabolite was desmethylfenitrothion (Sivori et al. 1999). In goats, small amounts of amino-fenitrothion residues were found in organs and tissues 1 day after exposure, while most of the metabolites were excreted through the urine, feces, and milk (Mihara et al. 1978). However, in another mammal, the red-backed vole, the main metabolite was the 3-methyl-4-nitrophenol (Tschaplinski and Gardner 1981). In some cases, metabolism is required to elicit toxicity from fenitrothion exposure. In cultures of

human neuroblastoma cells and hen brain homogenates fenitrothion did not inhibit acetylcholinesterase until incubated with either bromine or rat liver microsomes (Barber, Correll and Ehrich 1999).

Fenitrothion is promptly cleared from the body in vertebrates (Tschapinski and Gardner 1981; Tsuda et al. 1995), and only weak cases of bioaccumulation have been documented. In chicken exposed via ingestion, fenitrothion was detectable in whole blood, brain, liver, and abdominal fat, however none of these sites contained more than 0.09 ug/g (Trottier and Jankowska 1980). Likewise, cattle grazing on fenitrothion treated pastures demonstrated residues in fat stores post spray, but samples taken from the same livestock two weeks later showed no detectable amount of the pesticide in fat, muscle, or liver (Gilmour, McDougall and Spurgin 1999). The concentration of fenitrothion in the brain of the eel *Anguilla anguilla* was 9.6 times the plasma concentration while the fish were kept in water with pesticide present, but was completely eliminated upon transfer to pesticide-free water (Sancho et al. 1998). The fenitrooxon metabolite has also been investigated in the killifish and was eliminated even faster than fenitrothion, demonstrating biological half-lives of 6.3h and 2.3h respectively (Tsuda et al. 1997).

1.2.2 Effects of Fenitrothion in Field Trials

When sprayed over areas of forest, little pesticide is deposited on the forest floor or penetrates to the soil below, most landing and remaining on the overhanging foliage (Mallet and Cassista 1984). In the field, application to apple foliage demonstrated a rapid decrease in the concentration of fenitrothion dropping to 41-94% of the original concentration within one day (Chang-Yen, Nickless and Pickard 1983). Fenitrothion residues on the apple fruits did not penetrate beneath the peel (Barrio et al. 1995). In contrast, when applied to tomato plants under greenhouse conditions, after two weeks the parent compound accounted for the greatest percentage of recovered fenitrothion, followed by the *S*-methyl photodegradate on the leaf and fruit surfaces and a β -glucoside within the tissue extracts (Fukushima et al. 2003).

Fenitrothion degrades rapidly in aquatic environments, in the water as well as in sediment (Sudo, Kunimatsu and Okubo 2002; Maguire and Hale 1980). Fenitrothion in natural lake water has a half-life of 1.5-2 days; however in lake water transferred to the lab and kept in the dark the half-life was extended to 49.5 days (Greenhalgh, Dhawan and Weinberger 1980; Nishihara et al. 1997). Likewise, in soil environments the half life of fenitrothion has been reported as 2-3 days in soil and 1-2 days in sprayed pastures (Gilmour, McDougall and Spurgin 1999). While fenitrothion degrades similarly in organic soils versus sandy loam (Spillner, DeBaun and Menn 1979), mobility is increased with the amount of organic matter (Baarschers, Elvish and Ryan 1983).

1.2.3 Mechanism of Action

The principle toxic effect of fenitrothion is inhibition of acetylcholinesterase (Busby, Pearce and Garrity 1981; Durham and Ecobichon 1986), and subsequently, body tissues levels of free acetylcholine rise and acetylcholinesterase activity diminishes upon exposure (Trottier et al. 1980; Kobayashi et al. 1983; Nath and Kumar 1999). Initial inhibition is rapid (Escartin and Porte 1996) and can be observed in a number of tissues including the plasma, erythrocyte, and brain (Yoshida, Harada and Maita 1997). However, dependant on the species and its site preference within the habitat, full inhibition can be delayed, occurring as late as three days after exposure (Busby, White and Pearce 1991; Busby, Pearce and Garrity 1987). Several avian species observed after fenitrothion spray had brain AChE activities depressed in comparison to normal levels, including the Pine Siskin (*Carduelis pinus*; 76% inhibition) and the Yellow-rumped warbler (*Dendroica coronata*; 78% inhibition), which had lead to their subsequent mortality (Busby, White and Pearce 1991).

If the level of inhibition does not exceed a certain threshold (typically >50% for birds; Busby, Pearce and Garrity 1987 and references within), then the organism can usually recover under laboratory conditions. Freshwater field crabs (*Oziotelphusa senex senex*) dosed at three sublethal injections reached a peak inhibition of 50% 12 hours after dosing and began to recover after 24 hours (Bhagyalakshmi and Ramamurthi 1980). However, the recovery period following exposure is usually slow because fenitrothion binds to the enzyme irreversibly, requiring the synthesis of new enzyme. Thus, the

acetylcholinesterase of some species can remain inhibited for weeks after exposure (Bain et al. 2004; Hamilton, Hunter and Ruthven 1981).

1.2.4 Invertebrate Toxicity

Fenitrothion is equally as efficient at eradicating target pest insect species as it is other invertebrate populations. Exposure to fenitrothion inhibited acetylcholinesterase in honeybees (*Apis mellifera mellifera*) (>60%) when dosed with 0.2 nmol/bee (Bendahou, Bounias and Fleche 1999). Significantly, mortality of many species of non-target insects have been observed including backswimmers, *Anisops sardeus* (Hemiptera, Notonectidae) (24h LD50 16.7 ug/L) (Lahr et al. 2001, Lahr et al. 2000), the tenebrionid beetle *Trachyderma hispida* (24h LD50 9.64 ug/g) (Peveling and Demba 1997) and a few species of grasshoppers (Arthurs, Thomas and Langewald 2003). In addition to effects on adults, fenitrothion may also alter egg formation; in the moth *Spodoptera exigua* concentrations as low as 0.22 µg/larva/day for four days interfere with structural lipid formation (Fila, Adamski and Ziemnicki 2002). The toxicity of fenitrothion is just as efficient in aquatic environments. Exposed mussels (*Mytilus galloprovincialis* Lam.) demonstrated a reduction in glutathione in the digestive gland, muscle and gills, an indication of cell death and oxidative stress at a dose of 12 mg/L (Pena-Llopis, Ferrando and Pena 2002). A rotifer (*Brachionus*), a cladocera (*Moina*), and a copepod (*Mesocyclops*) also demonstrate toxicity to fenitrothion at low doses (24h LD50's 6.30, 0.05, 5.01 uL/L respectively) (Kaur and Ansal 1996).

1.2.5 Vertebrate Toxicity

One disadvantage of fenitrothion is its lack of species specificity and subsequent toxicity across taxa. In the bearded dragon lizard *Pogona itticeps* (Agamidae) plasma acetylcholinesterase levels were inhibited and remained so for 21 days following an exposure of 20 mg/kg (Bain et al. 2004). Red-backed voles (*Clethrionomys gapperi*) exhibited typical cholinergic symptoms when exposed at 516 mg/kg even though much higher doses were required for mortality (96h LD50 1330 mg/kg; Tschaplinski and Gardner 1981). In one of the few developmental experiments, rats exposed prenatally to 30 mg/kg fenitrothion had delayed ossification of sternum and cranium and decreased body weights and length. The frequency of early litter resorption and postimplantation losses also increased (Berlinska and Sitarek 1997). Declines in field populations of small mammals and desert reptiles have also been recorded after spray, possibly due to a decrease in available prey items and/or sublethal effects (Innes and Bendell 1989; Peveling et al. 2003).

In adult populations of the frog *Rana temporaria* L. while sizable quantities were required to cause lethality (LD50 ~2200 mg/kg), smaller doses lowered hematocrit and hemoglobin levels in the blood (Gromysz-Kalkowska and Szubartowska 1993). Densities of mink frogs in ponds in Canada contaminated with fenitrothion (sprayed at 210 g a.i./ha) were lower in areas of high frequency spraying (sprayed 3-4 times between 1987-90) when compared to areas of low frequency (not sprayed between 1987-90) (McAlpine, Burgess, and Busby 1998).

1.3 Fipronil

Fipronil, [(+/-)-5-amino-1-(2,6-dichloro- α,α,α -trifluoro-p-tolyl)-4-trifluoromethyl-sulfinylpyrazole-3-carbonitrile], is a phenyl pyrazole originally created in the 1980's by Rhone-Poulenc and introduced to the market in 1993 (Hamon, Gamboa and Garcia 1996; Colliot et al. 1992). Subsequently, it became registered in countries around the world, its main markets consisting of crop protection, specifically rice in the Asia Pacific region and maize in the Americas (Anon; Agrow 1997). Its uses include veterinary flea and tick treatment for domestic animals as well as the control of crop and turf pests (Atwell, Postal and Fitzgerald 1996; Jennings et al. 2002; Mehlhorn, Hansen and Mencke 2001). In agriculture, it was the first phenyl pyrazole insecticide introduced for pest management and the newest chemical in the second generation of pesticides (Moffat 1993), replacing the first generation consisting of the polychlorocycloalkanes, R-endosulfan and lindane (Hainzl, Cole and Casida 1998).

1.3.1 Chemical Properties

Overall, fipronil is poorly soluble in water (Table 1.1)

and preferentially binds to organic substances

found in soil (Aajoud,

Ravanel and Tissut 2003; Walse et al. 2004).

Fipronil applied to soil surfaces can break down

within hours under many circumstances, however

some substrate characteristics allow it to be

absorbed into soil and persist in the environment.

Table 1.1 Fipronil solubility (after Tingle 2003)

Fipronil half-lives in soil range from 44.5 hours to 533 hours (Ngim and Crosby 2001). Fipronil is non-polar and lipophilic and therefore accumulates in soils of high organic content (Mulrooney et al. 2003; Bobe, Coste and Cooper 1997). When it does so, fipronil and its desulfinyl and sulfone metabolites remain in the top ten centimeters of the soil and thus can easily come into contact with terrestrial organisms (Bobe et al. 1998). However, its sorption is lower in soils with low organic content and is further decreased in the presence of microbes (Ying and Kookana 2001, 2002). When applied to the surface of leaves, fipronil rapidly degrades, decreasing by 98% within 2 days after application on cotton (Mulrooney et al. 2003). In aquatic conditions, fipronil half-life ranges from 10.5 hours to 77.2 days (eg. in rice fields; Ngim and Crosby 2001; Shan et al. 2003). As in soil, the pH of the medium affects breakdown: fipronil is more stable in neutral and acidic solutions than in alkaline ones (Ramesh and Balasubramanian 1999; Bobe et al. 1998).

1.3.2 Fipronil Metabolites

Fipronil is metabolised to several major compounds, some of which are potentially more harmful than their parent compound (Figure 1.2; Fenet et al. 2001). Decomposition in sunlight, either on soil or leaf surfaces, rapidly produces the photodegradate desulfinylfipronil via the removal of the sulfur ion (Hainzl and Casida 1996; Bobe et al. 1998; Aajoud, Ravanel and Tissut 2003). Within soil organic matter, fipronil also degrades to a sulfone as a product of oxidation and addition of an oxygen moiety. The other two metabolites, the sulfide and amide, are products of reduction and hydrolysis, respectively, and are found in soils that are moist and/or have biological

activity. While the amide metabolite takes longer to form than the others, it is more polar, and thus is able to dissolve into aqueous media (Aajoud, Ravanel and Tissut 2003). In some species, exposure to the oxidative sulfone metabolite is more toxic than the parent compound, requiring less than half the amount that would be needed for fipronil to kill the same number of European corn borer larvae (Durham, Scharf and Siegfried 2001).

Likewise, the sulfide and sulfone metabolites are approximately three times more lethal to aquatic mosquito larvae than fipronil (24h LC_{50} 8.8 nM and 24.8 nM, respectively; Aajoud, Ravanel, and Tissut 2003). In addition, fipronil metabolites have longer half-lives than the parent compound. The desulfinylfipronil photodegradate is 10-fold more powerful at blocking mammalian, specifically murine, chloride channels than fipronil. In addition, when the parent compound was applied to the

Figure 1.2 Major metabolic pathways of fipronil (Tingle et al. 2003)

leaves of pea and pear plants exposed to sunlight, desulfinylfipronil comprised 45% of the total residues on the leaves 12 hours after application (Hainzl and Casida 1996). The main and almost exclusive *in vivo* metabolite is the sulfone derivative (Hainzl and Casida 1996; Scharf et al. 2000). In mice, this metabolite is detectable in liver, brain, kidney, fat and feces and remains in the liver and kidney at 24 hours post dose (Hainzl and Casida 1996). In the Western corn rootworm, the sulfone form is also the main metabolite, and the conversion is carried out by the cytochrome P450 pathway in the liver (Figure 1.3; Scharf et al. 2000; Durham, Siegfried and Scharf 2002). However, either the sulfone or the original fipronil molecule also has the potential for further breakdown by glutathione-S-transferase (GST) to any of several halogenated substitutions (Scharf et al. 2000).

Figure 1.3 Glutathione-S-transferase metabolism of fipronil in vivo (Scharf et al. 2000)

1.3.3 Mechanism of Action

Primarily, fipronil elicits neurotoxicity by blocking the gamma-aminobutyric acid (GABA) receptor within the nervous system of insects (Bloomquist 1996; Colliot et al. 1992; Gant et al. 1998). The effect on these chloride channels, measured via generated membrane currents (Ikeda et al. 2003), analysis of the GABA channel configurations (Grolleau and Sattelle 2000) and endogenous ligand binding, is striking and effective

(Caboni, Sammelson and Casida 2003). Suppression of the current across GABA channels in the rat nervous system is affected by fipronil in a dose-dependent manner (Figure 1.4; Ikeda et al. 2001).

Fipronil has a higher affinity to insect GABA receptors than mammalian receptors, a desirable trait for an insect pesticide.

Inhibition concentrations for 50% of the exposed receptors (IC_{50}) for insects range from 3-12 nM while the composite vertebrate IC_{50} for fipronil is 1103 nM (averaged from human, dog, mouse, chicken, quail, and salmon; Hainzl, Cole and Casida 1998). Fipronil was also shown to be 59 times more potent on cockroach GABA receptors than on rat GABA receptors and more toxic to the housefly than to mice in terms of lethality (24h LD_{50} 0.13 mg/kg and 41 mg/kg, respectively) and receptor inhibition

(IC_{50} 6.3 nM and 1010 nM, respectively) (Zhao et al. 2003; Hainzl and Casida 1996).

Figure 1.4 A: Inhibition of current by fipronil to control; B: Timeline of inhibition in a bath of 1 μ M Fipronil (Ikeda et al. 2001).

1.3.4 Invertebrate Toxicity

Fipronil is highly effective in producing high rates of mortality in insect populations (Kaufman, Scott and Rutz 2001; Ulloa-Chacon and Jaramillo 2003). It is used to control fleas on domestic animals, and is capable of killing one-hundred percent

of the population infesting an animal after 48 hours with very small amounts of chemical (McTier et al. 2003). Gel baits containing fipronil are used for household pests such as cockroaches and successfully achieve high rates of mortality at doses of 30 mg/m² treated area (le Patourel 2000; Durier and Rivault 2003). Toxicity to agricultural pests is also considerable (Herron, Rophail and Gullick 1996; Pedibhotla, Hall and Holmsen 1999), most relevantly in regards to desert locust hopper bands (Balanca and de Visscher 1997; Lecoq and Balanca 1998).

Although effective, fipronil has been criticized as taking longer to kill pests than other insecticides (APLC 1999; Kaakeh, Kaakeh and Bennett 1996). In addition, it has been found to be highly lethal to populations of beneficial insect groups such as termites, which aerate the soils of their environment and thus provide a crucial ecological service (Ibrahim, Henderson and Fei 2003; Peveling et al. 2003; Waite, Gold and Howell 2004). Populations of earthworms from the *Pheretima* group (Megascolecidae), which likewise aerate their habitat, experience weight loss and subsequent mortality after a spray application of 0.38 uL/ha (Mostert, Schoeman and van der Merwe 2000). Some of these affected insects are pest predators such as the insidious flower bug (*Orius insidiosus*), backswimmers (*Notonecta indica* L) and water scavenger beetles (*Tropisterrus lateralis*; Sparks et al. 1998; Studebaker and Kring 2003; Dennett, Bernhardt and Meisch 2003). Fipronil was also found to be lethal to three species of honey bees (LD50 0.004 – 1.130 ug/bee) and significantly decreased visitation to sucrose/honey feeders (*Brassica napus*) (Mayer and Lunden 1999). In France, honey bee health and subsequently honey production has been severely reduced

following the introduction of fipronil to maize and sunflower crops, and its role as an agricultural pesticide is under review by the French government (McKie and Paris 2004).

Studies investigating the effect of fipronil on aquatic organisms have observed significant lethality in grass shrimp, *Palaemonetes pugio* (96h LC₅₀ 0.32 µg/L), high mortality in a number of *Macrobrachium* shrimp species, and convulsions in the crab *Eriocheir sinensis* (Key et al. 2003; Shan et al. 2003). Rice farmers coat the outside hulls of pre-planted rice with fipronil as well as treating the plots once they are propagated to protect the crops from insect pests. This, in turn, may elevate the level of fipronil in the irrigation waters above the levels that are lethal to crayfish. In the United States, rice paddies are used for both rice cultivation and crayfish production and the toxicity to crayfish has been clearly identifiable: LC₅₀'s for red swamp crayfish (*Procambarus clarkii*) and white river crayfish (*Procambarus zonangulus*) were 14.3 mg/L and 19.5 mg/L respectively. In the same study, the fipronil sulfone (LC₅₀ = 11.2 mg/L), fipronil sulfide (LC₅₀ = 15.5 mg/L), and the photoproduct desulfinylfipronil (LC₅₀ = 68.6 mg/L) elicited high rates of toxicity: 60% mortality in contrast to 17% mortality in untreated water (Schlenk et al. 2001). Some aquatic species such as the crustaceans *Daphnia pulex* and *Eucypris virens* as well as the larval dipteran fauna, *Chaoborus crystallinus* demonstrate a marked sensitivity to concentrations of fipronil much lower than reported for *Procambarus* (48h LC₅₀ = 1478.5 nM (0.64 mg/L); Chaton et al. 2002). The toxicity to crayfish has provoked United States crayfish farmers to take legal action against Aventis Inc., the current producer of fipronil

products, even though representatives of the company claim the level of pesticide application is safe for crayfish (Biever et al 2003).

1.3.5 Vertebrate Toxicity

Fipronil LD50's have been determined for a very small range of nondomestic vertebrate species, mostly avian, and are highly variable, thus making prediction difficult for responses in vertebrate animals (Tingle et al. 2003, 2000). One of the few vertebrate toxicology studies conducted on a non-laboratory species was carried out on the fringe-toed lizard, *Acanthodactylus dumerili*. Lizards were exposed to 30 µg/g fipronil either directly via oral gavage or indirectly via ingested contaminated prey items (Peveling and Demba 2003). Mortality was significantly greater in the group fed prey items (62.5%) than those directly exposed (42.0%). It is important to note that lethality did not occur directly after exposure: the first incidence of mortality was observed a few days post exposure and commenced in the remaining group of lizards over a period of more than three weeks.

Sub-lethal effects of fipronil exposure on non-target species are largely unstudied, however some data do exist. Neurotoxic effects can occur, such as decreased hind leg splay in the rat at an acute dose of 5.0 mg/kg (USEPA 1996). In a separate study, female rats treated topically with an acute dose of 70 mg/kg had significantly higher plasma progesterone and significantly lower estrogen levels, in addition to a significantly longer estrous cycle when compared with controls (Ohi et al. 2004). The previously mentioned fringe-toed lizards receiving an acute dose of 30 µg/g

experienced a significant decrease in food consumption and body weight after exposure (Peveling and Demba 2003). Chronic exposure to fipronil also has toxic effects in mammals including neurotoxicity and reproductive dysfunction. Hereford heifer calves received a topical dose of 1 mL/10 kg of a 1% fipronil pour-on formulated solution (0.001 g/kg) approximately every seven weeks for 48 weeks. Treated calves experienced a consistently significant increase in body weight compared to controls for the duration of the study (Davey et al. 1999). The United States Environmental Protection Agency has conducted a battery of laboratory experiments investigating chronic fipronil exposure using a number of mammalian species, however details of these studies are often not reported (USEPA 1996).

In the veterinary field, domestic animals receiving spot or spray treatments of fipronil for flea and tick control may experience adverse reactions upon application, however the proportion of animals affected is very small (Tingle et al. 2000 and references within). In the United States in 1998 the number of reported cases of such reactions was only 334 for both cats and dogs (USEPA 1998b). In Australia, for the years 1996-2003, there was a total of 173 adverse reaction reports received by the Australian Pesticides and Veterinary Medicines Authority (APVMA) for humans, dogs, cats, and rabbits combined, an incidence rate of <0.01% (Australian Pesticides and Veterinary Medicines Authority 2003).

See Appendix 1 for a composite table of the toxicity of fipronil to a selection of invertebrate and vertebrate species.

1.4 Thyroid Function and Pesticides

1.4.1 Normal thyroid function

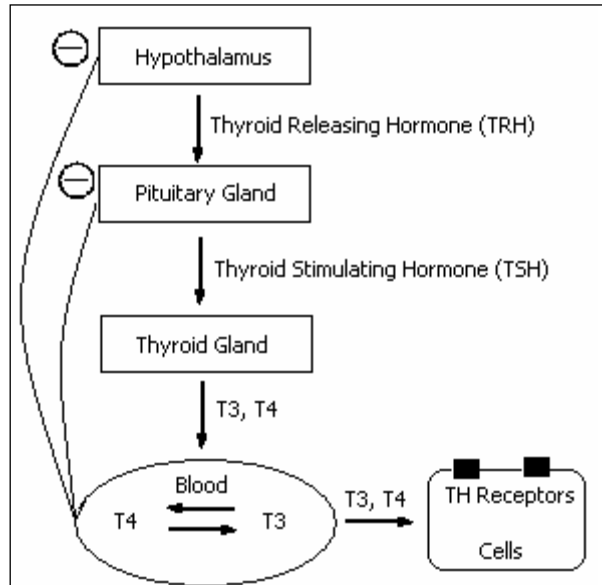


Figure 1.5: Hypothalamic –Pituitary Thyroid Axis and related hormones

The thyroid system, across vertebrate taxa, strongly influences a wide range of bodily processes including growth, metabolism, and aspects of reproduction and behaviour (Dussault and Ruel). Thyroid hormones triiodothyronine (T3) and thyroxine (T4) are important for creating proper muscle composition and maintenance (King and King 1978; King, King

and Jacaruso 1981; King and EntriKin 1991). In American tree sparrows (*Spizella arborea*) the timing of testes enlargement and molt associated with breeding appears to be dependent on thyroid status (Wilson and Reinert 1996). Circulating T4/T3 levels are strongly correlated with metabolism and processes associated with it, such as thermogenesis, metamorphosis, and osmotic balance (Rattner, Becker and Nakatsugawa 1987; Tomasi, Ashcroft and Britzke 2001; Collin et al. 2003). In altricial birds, thyroid development, specifically size, growth, and follicle size, has been linked with the ability to thermoregulate as the juvenile birds mature (Olson et al. 1999). Control of thyroid hormone is accomplished via the hypothalamus-pituitary-thyroid axis (Figure 1.5). The hypothalamus produces thyroid releasing hormone which stimulates the pituitary to

produce thyroid stimulating hormone which in turn prompts the thyroid to produce thyroxine (T4). Both the hypothalamus and the pituitary are subsequently influenced by the circulating plasma levels of thyroxine (T4) and its product triiodothyronine (T3) via a negative feedback loop (Figure 1.5).

Most interestingly, thyroid hormones influence the cellular structure and function of glutathione S-transferase (GST) as well as monoamine oxidase (MAO), enzymes capable of metabolizing fipronil *in vivo* (Grippo and Fernandez 1977; Coecke et al. 2000; Scharff et al. 2000). An introduced dose of triiodothyronine, thyroxine or 17 β -estradiol *in vitro* reduces GST activity in rat liver cells and hypothyroidism decreases MAO activity *in vivo* (Grippo and Fernandez 1977; Coecke et al. 2000). Therefore, if fipronil disrupts the effects of these hormones, it may indirectly interfere with its own *in vivo* decomposition.

1.4.2 Thyroid function and development

Normal thyroid function is particularly important during development and is critical temporally and spatially for formation of normal bodily structures and functions. Within the nervous system, T4 and T3 play an essential role in regulating neuronal growth and brain formation (Koibuchi and Chin 2000). The brain is rich in intracellular triiodothyronine receptors and during development adequate levels of thyroid hormone secretion are critical to attain normal levels of cell differentiation, dendritic and axonal growth and migration, myelination, gene expression and synaptogenesis (Bernal and Nunez 1995; Oppenheimer and Schwartz 1997). Offspring of hypothyroid mothers

have a drastic underdevelopment of these structures that is not always corrected by the onset of fetal thyroid hormone production (Oppenheimer and Schwartz 1997; Singh, Upadhyay and Godbole 2003). Similarly, hyperthyroid mothers also produce offspring with altered neural development, such as a deficit in myelination of the nervous tissue in the brain (Marta et al. 1998).

1.4.3 Xenobiotics and Thyroid Hormones

The effects of xenobiotics on thyroid hormone levels are diverse and have not been studied to the same degree as endocrine disrupters affecting reproduction. Comprehensive reviews of these substances exist (Brucker-Davis 1998; Zoeller 2003) and, thus, only a couple examples will be provided here. In Florida, researchers noted that alligators (*Alligator mississippiensis*) from lakes chemically contaminated with a mixture of substances, including polychlorinated biphenyls and organochlorine pesticides, had levels of plasma T4 significantly higher than levels in alligators at pristine lakes. In addition, the researchers noted that while alligator size is normally correlated with T4 concentrations, contaminated populations lacked a clear relationship between size and thyroid hormone levels (Crain et al. 1998; Gunderson et al. 2002). American kestrels (*Falco sparverius*) were exposed to Aroclor 1242, a mixture of PCB's, at either 6 or 60 ppm in the diet for five months. Females demonstrated a significant level of depressed plasma thyroxine while males followed a similar but not statistically significant decrease (Quinn et al. 2002).

Thyroid disrupting chemicals can compete for receptors or alter processes normally mediated by thyroid hormone. For example, halogenated aromatic compounds compete with thyroxine for the T4 transport protein, transthyretin (Sandau et al. 2000). Organochlorine exposure in humans decreases plasma triiodothyronine (T3) concentrations, depresses resting metabolic rate and reduces fat mobilization (Pelletier, Imbeault and Tremblay 2003). An increase in hepatic metabolism of thyroxine stimulated by xenobiotic exposure can lead to an overall decrease in plasma thyroxine concentration (Tomasi, Ashcraft and Britzke 2001). Lower T4/T3 levels are often correlated with hyperplasia or hypertrophy of the thyroid gland as a result of activation of the HPT axis, resulting in an increase in thyroid organ weight and incidence of tumors (Catena et al. 2003; Wilson et al. 1996).

In a review of studies (mostly unpublished USEPA studies, Office of Pesticide Programs) concerning the relationship between pesticides and thyroid carcinoma, it was noted that fipronil altered the structure and function of the thyroid gland in rats following fifty weeks of chronic treatment (Hurley, Hill and Whiting 1998). Unfortunately, details of this study, including the dose, were not specified in the review. In the aforementioned study, fipronil treated rats showed significantly decreased T4 and increased thyroid stimulating hormone (TSH) in the plasma. In addition, significantly increased iodide uptake by the thyroid gland and increased thyroid peroxidase activity were observed, indicating increased synthesis of thyroxine. It was concluded that fipronil increased clearance of serum thyroxine via increased liver metabolism of the compound, resulting in the decrease in plasma T4 concentrations (Hurley, Hill and

Whiting 1998). This decrease in turn stimulated the hypothalamus to produce and secrete more TRH and the pituitary gland to produce and secrete more thyroid stimulating hormone. Thyroid gland hypertrophy and increased thyroid gland weight were also noted in this review (Hurley, Hill and Whiting 1998).

1.5 Maternal Transfer of Fipronil

Disruption of normal thyroid function by fipronil has the potential to affect developing offspring via two distinct methods. The first is an alteration of maternal thyroid function which in turn affects the development of the embryo. In oviparous organisms, thyroid hormone is deposited within the egg at concentrations correlated with maternal plasma T3 and T4 concentrations (Wilson and McNabb 1997). This quantity will account for the embryo's total supply until its own thyroid develops and begins hormone production (approximately developmental day 6 in chickens; Norris 1996). Thyroid hormones acquired from the mother are essential for proper development as previously described. Thus, altered maternal thyroid hormone levels can have great impacts on embryo maturation if the amounts provided are significantly low (Pickard et al. 1999).

The second manner in which fipronil can disrupt development is by directly affecting the embryo. The parental generation is capable of incorporating contaminants into developing eggs in conjunction with the transfer of lipoproteins or hormones from mother to egg (Vieira et al. 1995; Wilson and McNabb 1997; Matsushita et al. 2002).

This *in ovo* exposure can lead to downstream effects on the offspring that may differ extensively from those observed in adults, (Metcalf et al. 2000; Furusawa and Morita 2000) perhaps due to processes carried out exclusively in the egg (Scharff et al. 2000). Contaminants initially introduced to the embryo from the mother may accumulate in the mother and the fetus at different concentrations and thus elicit differing magnitudes of toxicity. In female mice exposed to PCB's, the largest concentration was accumulated in the maternal fat, however there was large variation in the concentrations between tissues assayed when readings were taken 4 days post-acute dose of 2 umol/kg (Darnerud, Sinjari and Jonsson 1996). In addition, embryonic effects may be subtle or may only be evident after varying periods of development (Hayward and Wingfield 2004; Fernie et al. 2001; York et al. 2001).

Makris and others (1998) has shown that fipronil can elicit teratogenic effects in progeny when administered to pregnant rats (doses of 0.05, 0.9 and 18.5 mg/kg/day from gestational day 6 to post natal day 10). In this study, the F1 generation suffered from high mortality; those that did survive had reduced body and brain masses accompanied by stunted learning and memory skills, a decrease in sensory function, and diminished auditory startle habituation (Makris et al. 1998).

1.6 Developmental Risk and Locust Control pesticides: the present study

In Australian rangeland, the avian pre-breeding period corresponds with seasonal

periods of food i.e. insect
abundance (Edwards, Jones

and Davies 2002; Rooney

et al. 2003; Orlando et al.

2003). The conditions that

foster the increase in insect

populations also allow for

increases in locust

populations and activity. Subsequently, pesticide spray events correspond with

reproductive activities in vertebrates. Vertebrate populations that feed on insects are

attracted to the areas of high locust abundance, thus increasing the risk of pesticide

exposure.

Table 1.2 Lethal Dose 50s of fipronil to a variety of avian species (Hanon et al. 1996)

Adult birds exposed to fipronil have the potential to sequester the chemical in their fat stores because it is lipid soluble. This lipophilic xenobiotic may then travel with these lipids and gain deposition in the egg yolk. Thus, fipronil-exposed females are at risk of sequestering these chemicals within their eggs, thus exposing the embryo to this pesticide throughout development. In previous studies, of gallinaceous birds, such as quail, pheasant, and partridge were highly sensitive to fipronil (Table 1.2 from Hamon et al. 1996). In contrast, Zebra finches were tolerant to high doses of fipronil,

the LD₅₀ exceeding 1200 mg/kg (Fildes, unpublished data). Therefore, to test the sublethal effects of fipronil on developing embryos it is desirable to use eggs of a species known to be sensitive to fipronil, such as the domestic chicken, so that there is an increased chance that effects will be observable at low doses. In contrast, to test the amount of fipronil that is passed from the mother's blood to the egg yolk, it is desirable to use a species that is resistant to fipronil toxicity, such as Zebra Finches, because a large amount of pesticide can be administered to the mother, increasing the likelihood that the chemical will be detectable in the egg.

While to date no *in ovo* studies have investigated the developmental effects of fipronil, studies investigating fenitrothion administered *in ovo* topically and via injection have been conducted. In fenitrothion treated chicken eggs, acetylcholinesterase inhibition was observed to reach values as high as 80% at doses of 100 mg/kg (Farage-Elawar and Francis 1987). Other observations included a decrease in growth and an alteration of gait demonstrated by the curling under of toes (Farage-Elawar and Francis 1988a and b). White leghorn chicks experienced a decrease in hatching success and those that did hatch displayed stunted growth as well as deformed legs and gait when exposed in the egg (Paul and Vadlamudi 1976). Fenitrothion residues are detectable in egg yolk up to twelve days post exposure and the parent compound is capable of crossing the egg shell (Varga et al. 2002). Thus the developing embryo may undergo extended exposure via compound deposited in the egg via maternal transfer as well as external environmental residues.

1.6.1 Study Aims

Given the information above the aims of this study are:

1. To examine the consequence of pesticide exposure on a developing embryo by delivering a dose of either fipronil or fenitrothion directly into the yolk of an avian egg early in development.
2. To measure the effect of pesticide exposure on particular variables associated with growth and development.
3. To measure the potential for fipronil to affect thyroxine hormone status and physical traits known to be affected by thyroxine.
4. To measure the effects of fenitrothion on cholinesterase activity of post-hatching chicks.
5. To determine the relative amount of fipronil that is transferred from a laying female to her eggs in an Australian native species, the Zebra finch (*Taeniopygia guttata*) dosed with variable amounts of fipronil.

This study provides data on the developmental effects of fipronil and fenitrothion, which currently are lacking. To date no *in ovo* study has been conducted utilizing fipronil and those that exist regarding fenitrothion *in ovo* did not investigate its effects on oxygen consumption.

Chapter 2

Methods Development and Final Study Design

2.1 Introduction

Plague locusts can aggregate over vast areas in eastern Australia in a variety of habitats, and therefore many native species are potentially at risk of exposure to fenitrothion and fipronil during control operations. In the Australian semiarid zone avian breeding is irregular and typically occurs after rainfall that increases food abundance, especially insect populations (Edwards, Jones and Davies 2002; Orlando et al. 2003; Rooney et al. 2003). The nature of locust control operations is such that pesticides are sprayed from the air over large bands of young locusts that aggregate and move in high densities. Aggregations of locust nymphs in semi-arid habitats provide an unusually abundant food source for breeding birds. By ingesting the sprayed locusts or by coming into contact with sprayed vegetation, a breeding bird may be exposed to the pesticides. Subsequently, incorporation of these chemicals into the egg has the potential to alter embryonic development.

This study was undertaken to examine the possible effects of fipronil or fenitrothion on developing embryos of an oviparous species. The *in ovo* avian model is commonly used as a test system for assessing teratogenic effects of contaminants (Berg et al. 1999) and is particularly attractive because a known amount of contaminants and specific metabolites can be delivered precisely.

The first aim of this study was to simulate maternal transfer of fipronil or fenitrothion into the yolk of an avian egg, as early in development as possible. In addition, given fipronil's suspected effect on thyroid status and fenitrothion's demonstrated inhibition of cholinesterases, it was especially important to measure their effects on growth and development. If such effects or others that impair embryo health or development occur, it was predicted that embryo oxygen consumption would provide a good general indicator. Teratogenic studies of the effect of fenitrothion administered *in ovo* topically and via injection have been conducted previously. These have shown significant inhibition of embryo plasma cholinesterases as well as decreased body masses in hatchlings (Paul and Vadlamudi 1976; Farage-Elawar and Francis 1988). However, no studies have yet examined the effects of fenitrothion on embryonic development of oviparous animals. Similarly, studies examining the developmental effects of fipronil on avian embryos have not been reported.

A pilot study was carried out to develop effective methods for injecting fertile eggs with oil-based compounds as well as for measuring metabolic and body morphometric data. Injections took place on day 0 of development prior to any incubation and chicks were then killed one week post-hatch. The prehatching mortality from the first set of eggs was unexpectedly high ($78.6\% \pm 7.528$) across all treatments resulting in sample sizes inadequate for statistical analysis. In addition, the OIL and CONTROL treatment groups experienced surprisingly similar low hatchability (33% and 25%, respectively) to the pesticide treated groups, indicating that there might be

mortality across groups due to methodology rather than treatment effect. Thus these methodological problems prompted a closer look at certain aspects of the study design

Subsequently, a series of pilot experiments was conducted to investigate the effects of different techniques and control substances on hatchability. These pilot studies tested hole creation and injection methods, the type of vehicle, and the day of injection relative to embryonic development. Mortality was assessed for up to ten days following injection and compared between methods to identify those with the least mortality to control embryos. Described in this chapter are general methods used throughout all experiments in terms of egg incubation and monitoring, the methods and results of the pilot studies and how the information gathered from them influenced the final experimental design and, subsequently, the final study design.

2.2 General Handling Procedures

Certain procedures regarding general egg incubation and treatment were the same throughout all the experiments and are described below. Procedures unique to each of the studies are described later in their respective sections.

2.2.1 Test Subjects

Eggs of the common chicken, *Gallus domesticus*, obtained from Ingham's Nursery (Casula, NSW), were weighed and randomly divided into treatment groups. Beginning on Day 4, eggs were candled and weighed every other day until hatching.

Eggs were considered infertile when there was a complete absence of vascularisation or embryonic development and were discarded. Fertile eggs and embryos that suffered mortality prior to hatching were frozen as soon as detected and the time and date noted.

2.2.2 Incubation of Eggs

Eggs were incubated in an Octagon 40 self-rotating incubator starting on Day 0 of development (Brinsea Products Ltd., Sanford, N. Somerset, UK) at 37°C continuously until they hatched approximately 21 days later. Eggs were weighed every other day but were not away from the incubator for longer than a few minutes. The incubators were kept in the University of Wollongong Animal House in a constant temperature room a 12:12 light:dark photoperiod with 15 min dawn/dusk. The incubator humidity was maintained according to the manufacturer's instructions. That this provided adequate humidity was verified by determining water conductance mass loss: eggs lost an average of 14-15% water over the incubation period, typical for normally developing chicken embryos (Rahn, Paganelli & Ar, 1974).

2.2.3 Treatments

Pesticide doses were made up in grapeseed or canola oil and were administered on the basis of dose/kg whole fresh egg. These injection volumes for all doses were approximately 50µL given an average egg weight of 65 g. The final doses of fipronil (FIP) or fenitrothion (FEN) were based on a logarithmic scale to examine the potential effects of a wide range of doses. Eggs receiving an oil (OIL) vehicle alone and eggs incubated without any injection (CONTROL) were included in each trial. The latter

was to account for both intrinsic and extrinsic influences on hatchability of unaltered eggs which served as an overall baseline for all other treatments.

2.2.4 Injection

Each egg was candled prior to injection to identify the embryo's position within the egg and a mark was made on the opposite side of the egg. The shell surface at this mark was wiped with 70% ethanol and a small hole was made through the shell using a 1 mm drill bit. The egg was held against the candling lamp while the treatment solution was injected directly into the egg yolk through the hole using a new 26 gauge $\frac{3}{4}$ " needle for each egg and a 50 μ L Hamilton glass syringe. The needle was always inserted the full $\frac{3}{4}$ " into the yolk to attempt to inject the pesticide at the same position in the yolks of all eggs. The syringe was cleaned with ethanol between injections of different treatments. Holes made in the egg were sealed with Elvax (an inert wax composition; Jerry Stewart Enterprises, California) heated to liquid on a hot plate. Once the wax had hardened (approximately 30 seconds), the eggs were placed back in the incubator.

2.2.5 Post Hatching Procedure

Hatched chicks were given a colour band on one leg and placed in an enclosure with food, water and a heat lamp. Chicks unable to feed/drink were manually assisted. Overall hatchability as well as time of hatching \pm 5 hours was recorded.

2.3 Pilot Studies

Following the initial study described earlier, a series of smaller studies was conducted to identify procedures to improved overall hatching success. There were four

separate trials performed to investigate the influence of type of oil used as well as overall injection methods on egg hatchability.

2.3.1 Pilot Study Methods

Treatments consisted of an injection of grapeseed oil (GP; 50 μ L/egg), canola oil (CA; 50 μ L/egg), piercing the egg shell with a needle but not injecting a test substance (SHAM), creating a hole in the egg shell with a drill bit by hand but introducing nothing to the egg interior (HO), creating a hole with an electric drill but introducing nothing to the egg interior (DR), and the CONTROL treatment. In this series of tests, the DR treatment group was the only one for which a motorized drill was used; all other holes were made by hand using the drill bit. The oils used were purchased the same day as a given trial and opened fresh at the time of injection. In three of the four studies, injections took place on Day 3 of development. However, in the fourth study a group of eggs received treatments of CA, HO, or DR on developmental Day 0 to examine the effect of earlier exposure on hatchability.

2.3.2 Pilot Study Results

Eggs treated on Day 0 of development had lower mortality than embryos with corresponding treatments on Day 3. By Day 6 of development the CA treated group treated on Day 0 had almost 10% more viable eggs than when the injection was administered on Day 3. However, the CONTROL treatment group experienced an unexpected 14% decline in viability not seen in the other pilot studies. Because this group received no injection, the drop in viability is due to some other unknown factor.

Overall the GP treated group experienced proportionately lower viability as early as four days post dose in comparison with the CA treated group (71% and 92% respectively) and by ten days post dose, the CA treated group had almost 20% more viable eggs remaining. The SHAM treated group experienced viability approximately 40% lower than that of the CONTROL treated group, indicating that the creation of the hole in the shell caused some level of disturbance. However, the SHAM treated group had viability almost identical to the CA treated group, signifying that after creating the hole in the egg, the introduction of oil control vehicle added little to no additional disturbance. The eggs pierced with a Dremmel drill experienced a reduced viability across incubation days when compared to those that received a hole using a drill bit by hand (Day 4, 74% and 84% respectively).

2.3.3 Pilot Study Discussion

Sample Size:

It was important that the resulting sample size post treatment was large enough for statistical analysis. Previous *in ovo* studies investigating contaminant effects have varied greatly in their choice of injection day (Day 0: Lipsitz et al. 1997; Day 3: Boily, Ndayibagira and Spear 2003; Day 4: Brunstrom and Lund 1988; Day 14: Annas et al. 1998; Day 15: Farage-Elawar and Francis 1988). Injection on Day 0 of development would have been optimal in order to most closely mimic the mechanism of maternal transfer. However, in the pilot studies, there were an unexpectedly high and variable number of infertile eggs in our pilot treatment groups ranging from 0 to 7.3%. A more

robust sample size could be obtained when infertile eggs were removed prior to treatment. Thus, injections of the final experiment were administered at Day 3, when fertility could be determined, and only fertile eggs were included in treatment groups. In addition, the number of eggs that could be processed at one time was limited due to incubator size and the time allotted for metabolic measurements during the light period. Therefore the injection of treatments was carried out in rounds; each round of experiments contained both controls (CA and CONTROL) and a few different treatments to account for temporal variation between injections.

Oil Vehicle:

Because both FEN and FIP are non-polar, it was necessary to use a lipid-based vehicle. In the initial pilot study grapeseed oil was chosen because it had been used in earlier oral dosing studies (Fildes, unpublished data). When compared with canola oil as the injection vehicle, the mortality observed for the grapeseed oil group was higher than for the canola oil groups (Day 4, 21%; Day 10, 18%). Thus, in the final study design canola oil was used for the pesticide vehicle as well as for the control injection.

Injection Conditions:

In the present study a high speed Dremmel drill with a sharp 1 mm drill bit was used to create a discrete hole without fracturing the shell. It was hypothesized that the vibration of the drill may have disturbed the embryo, thus the viability of eggs with power drill versus hand drilled holes were compared. Eggs that had a hole created by the power drill experienced 44% viability compared to 60% viability at day 10 in those

that had the hole created by hand. Thus, for the final study design all injection holes were made by hand-twisting the drill bit.

2.4 Final Study Design

2.4.1 Final Study Methods

Egg Selection:

Thirty-six to sixty eggs were obtained for each round of injections. Those eggs that fell outside of one standard deviation of mean egg mass (66.60 ± 4.59 as determined from a total of 489 eggs previously weighed on Day 0) were discarded in order to minimize the effects of egg size on viability. Eggs were also candled on arrival and those with shells considered to be overly mottled indicating variation in shell deposition were discarded. A mottled shell makes the embryo within difficult to see thus affecting the assessment of fertility. In addition, uneven shell thickness affects the conductance of gases between the embryo and the environment and may affect measurements of oxygen consumption and rates of water loss (Wagner-Amos and Seymour 2002).

Treatments:

Treatments were carried out over two trials, with the injections of the first trial occurring in May 2004 and the injections of the second trial occurring August-September 2004 (schedule, Table 2.4). Data were briefly analysed following the first trial to ensure the changes in methodology were appropriate. Within each trial, eggs

were injected in rounds with 40-60 eggs per round. Each round of injections was approximately a week apart and consisted of injections of 4-12 different treatments (Table 2.4).

Table 2.4 Injection schedule for eggs of the domestic chicken that received a single treatment of fipronil, fenitrothion (mg/kg) or canola oil vehicle (50 µL/egg) *in ovo*. TMT – treated; DIS – discarded (includes eggs that were mottled, infertile, or outside weight range).

Treat-ment	Round 1: 8 May 04	Round 2: 20 May 04	Round 3: 27 May 04	Round 1: 16 Aug 04	Round 2: 30 Aug 04	Round 3: 6 Sept 04	Round 4: 10 Sept 04	Round 5: 17 Sept 04	Total Injected
Fip 1.13 X10 ⁻⁷	7	2	0	0	0	0	0	11	20
Fip 1.13 X10 ⁻⁶	7	2	0	0	0	8	7	0	24
Fip 1.13 X10 ⁻⁵	7	2	0	0	0	7	7	0	23
Fip 1.13 X10 ⁻⁴	7	2	0	6	9	0	0	0	24
Fip 1.13 X10 ⁻³	7	2	0	6	9	0	0	0	24
Fip 1.13 X10 ⁻²	8	1	0	0	0	0	0	0	9
Fen 0.001	8	5	4	0	0	0	0	11	28
Fen 0.01	0	5	4	0	0	8	7	0	24
Fen 0.1	0	6	4	0	0	6	7	0	23
Fen 1.0	0	5	4	6	8	0	0	0	23
Fen 5.0	0	5	4	0	0	0	0	0	9
Fen 10.0	0	0	0	6	7	0	0	0	13
Oil	8	0	4	6	3	3	4	2	30
Control	8	2	9	6	4	3	4	1	37
TOTAL	60 (59 TMT, 1 DIS)	40 (39 TMT, 1 DIS)	40 (33 TMT, 7 DIS)	36 (36 TMT, 0 DIS)	48 (40 TMT, 8 DIS)	48 (35 TMT, 13 DIS)	48 (36 TMT, 12 DIS)	48 (25 TMT, 23 DIS)	311

Injection:

Injectons took place after three days of incubation four to six eggs at a time.

Each treatment was split in half, with one half the eggs put in incubator 1 and the other half in incubator 2, to control for possible incubator malfunction.

Post Mortem Assessments:

Chicks were weighed and then euthanized 24 hours post-hatch using CO₂ inhalation. Once dead, blood was collected into heparinized capillary tubes following puncture of a jugular vein. The blood was then spun in a hematocrit centrifuge for 5 minutes, and the plasma was transferred to Eppendorf tubes and stored at -20°C until assayed for cholinesterase activity (FEN treatments) or thyroxine (FIP treatments). The length of the tarsus and skull of the chicks were measured to the nearest 0.01 mm using Vernier calipers (Multitoyo, Japan). The brain was removed and frozen at -20°C.

2.4.2 Conclusions

The preliminary experiments permitted the methods to be refined and procedures best suited to the study objectives to be selected. This knowledge was incorporated into a protocol used to evaluate the effects of fipronil or fenitrothion on parameters of growth and development in the domestic chicken *in ovo*. For all chicks, any effects of pesticide on body mass and skeletal growth as well as hatchability were investigated. In particular, the impact of fipronil on thyroid status and the effect of fenitrothion on cholinesterase inhibition were assessed. Lastly, the effects of both pesticides on metabolic rate *in ovo* were measured.

Chapter 3

Fipronil: Hatchability, Post-hatching Morphometrics and Thyroid Hormone Status

3.1 Introduction

Fipronil is a phenyl-pyrazole widely used in the control of pest species domestically and agriculturally. It acts as a γ -aminobutyric acid (GABA)-gated chloride channel inhibitor eliciting toxicity via nervous system interruption (Bloomquist 1996; Bloomquist 1993; Cole, Nicholson and Casida 1993; Grant et al. 1998). The lethal effect of fipronil is highly variable between species, therefore toxicity to any species is difficult to predict based on the effect of fipronil on another species (Tingle et al. 2003). The molecule is small and lipophilic and moves across cell membranes easily. Fipronil concentrations within the organism greatly exceed levels expected from chemical diffusion alone as it collects in lipid filled areas (Chaton et al. 2001). Fipronil molecules can thus bioaccumulate in the fat stores of exposed organisms (Hainzl and Casida 1996). For example, stripe-faced dunnarts (*Sminthopsis macroura*) collected from areas that had been aerially sprayed with fipronil, had blood levels of fipronil residues as high as 44.7 $\mu\text{g/kg}$ several months after spray (P. Story, personal communication).

The hypothalamic-pituitary-thyroidal axis (Chapter 1) regulates centres in the brain sensitive to circulating levels of T4 and T3. Blood levels of these hormones

provide feedback that regulates their production by the thyroid, keeping plasma concentrations within a tight range. Thyroid hormones are especially important during development as they are required to induce stages of growth (Power et al. 2001) and generate essential physiological processes during short windows of development (Olson et al. 1999). Avian thyroid hormones and the hypothalamic-pituitary-thyroidal axis overall is similar to that of mammals in their structure and regulation (Epple and Stetson 1980), although functional chronology varies, as described below.

Birds can be divided into two main categories based on their modes of development. Altricial species complete a portion of their development within the egg and the remaining growth and maturation *ex ovo* in the nest. They emerge from the egg incapable of thermoregulation or locomotion, lacking feathers or sight, and are thus extremely dependent on parental care (Starck and Ricklefs 1998). Precocial birds, by contrast, complete their embryonic development within the egg and emerge at a well-developed juvenile stage. With respect to thyroid development, altricial birds hatch with low thyroid activity (McNabb 1992; McNabb and Cheng 1985; McNabb, Scanes and Zeman 1998), which becomes more active within days (Olson et al. 1999). Precocial birds, such as chickens, have detectable levels of thyroid hormones as early as 4.5 days of *in ovo* development (Thommes 1987), and have a fully functional hypothalamic-pituitary-thyroidal axis by embryonic Day 11 (Thommes et al. 1977). Thus, because precocial birds complete their thyroidal development solely within the egg, disturbances of thyroid status *in ovo* may produce more dramatic and observable dysregulation of

normal thyroid function, making them an excellent species to study for thyroid disrupting chemicals.

While still a relatively unexplored field, there is increasing evidence that certain environmental contaminants alter normal thyroid function (Brucker-Davis 1998). When administered to adult American kestrels chronically via the diet, polychlorinated biphenyl (PCB) mixtures caused a significant decrease in plasma triiodothyronine (T3) levels (Smits et al. 2002). Alternatively, while an acute dose of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in adult great blue herons had no effect on T3 levels, it did significantly elevate plasma thyroxine concentrations (Janz and Bellward 1997). In developing animals, abnormal thyroid function following contaminant exposure may lead to subsequent alterations of growth and development. Rat pups (21-days old) treated with PCB 118 during gestation (Days 10-16), had significant decreases in both plasma thyroxine concentrations and body weight (Ness et al. 1993).

Developing embryos risk exposure to environmental contaminants via transfer from the mother. Contaminants initially introduced to the maternal body may accumulate in maternal fat stores at different concentrations and, thus, varying amounts of xenobiotics can be transferred to the offspring. In mice dosed with dioxin-like PCB congeners, a majority of contaminant was sequestered in the maternal fat resulting in an average transfer rate from the mother to offspring of 16.2% (Darnerud, Sinjari and Jonsson 1996). Likewise, American kestrels fed 5-7 $\mu\text{g/g}$ bird of PCB's per day for 100 days passed on these PCB's to their eggs at an average concentration of 34.1 $\mu\text{g/g}$ egg, amounting to 7% - 5% of the total maternal load (Ferne et al. 2003). Finally, other

contaminants are selectively transferred to the offspring in greater proportions than that remaining in the mother, thus the offspring receives a majority of the maternal dose. For example, frogs exposed to PCBs or organochlorine pesticides produced eggs with concentrations of these chemicals that were at least twice as high as maternal concentrations (Kadokami et al. 2004). Brown house snakes administered selenium at 20 µg/g produced hatchlings with concentrations >24 µg/g, most likely due to maternal bioaccumulation (Hopkins et al. 2004). To date no maternal transfer study has been published to determine transfer of fipronil into yolk. Because the proportions transferred maternally can vary between pesticides and species, it is important to measure specifically the amount of fipronil transferred from mother to egg in comparison to the amount originally administered to the mother. Knowing the relationship between maternal dose of fipronil and the resultant embryonic dose is needed to predict the probability of embryo intoxication following adult exposure. This has specific implications for Australian native species living in areas where fipronil is used for pest control.

Developmentally, fipronil has been shown to cause teratogenic effects in mammals. When administered to pregnant rats, the progeny demonstrated decreased body and brain weights, stunted learning and an overall decrease in survival (Makris et al. 1998). However, no developmental studies have been conducted in oviparous animals. In addition, limited evidence suggests that fipronil may adversely affect normal thyroid function in rats (USEPA 1996; Makris et al. 1998; Hurley, Hill and Whiting 1998). Specifically, it has been claimed that fipronil altered the thyroid status

in rats following fifty weeks of chronic treatment (Hurley, Hill and Whiting 1998). A decrease in thyroid hormone and an increase in thyroid stimulating hormone (TSH) in the plasma were observed in conjunction with a subsequent increase in thyroid gland weight.

Given the thyroid's effects on growth and development and fipronil's potential disturbance of these processes, one would expect the potential for alterations in development following exposure to the pesticide. In particular, regular thyroid function is crucial for muscle and bone development (Wilson and McNabb 1997) and offspring that are hypothyroid display defects in these structures (Deaton, Bishop and Butler 1998; King et al. 1981; Kaneshige et al. 2000). In addition, plasma thyroid hormone levels have been used in the past to determine thyroid status in avian species during normal development (Olson et al. 1999) as well as following pesticide exposure (Gould, Cooper and Scanes 1999 and citations within). Thus, if fipronil affected the thyroid in developing chicks, one might expect to see growth abnormalities, in particular the bones, as well as irregular plasma thyroid hormone levels. In birds, overall size can be accurately estimated using skeletal measurements, with the tarsus representing the most reliable univariate estimator of all skeletal components (Freeman and Jackson 1990). Lastly, by directly measuring the amount of fipronil in the eggs of breeding females who were administered a known dose, the amount of transfer from mother to embryo can be determined.

3.2 Methods

3.2.1 Egg Treatment

I followed procedures for injection, egg handling, and body measurements as described in Chapter 2. Treatment solutions were made up in canola oil and were formulated so that the injection volume was 50 μL for each egg. Eggs were treated with fipronil (FIP) or oil vehicle only (OIL); there were 6 fipronil treatments (Fip 1.13×10^{-2} , Fip 1.13×10^{-3} , Fip 1.13×10^{-4} , Fip 1.13×10^{-5} , Fip 1.13×10^{-6} , and Fip 1.13×10^{-7} mg/kg egg). A control group incubated without any injection was also included (CONTROL). Fipronil (>98% pure) was obtained from Aventis Crop Science (Lyon, France). Eggs were examined three to four times daily to determine date of hatching and overall hatchability of a given treatment, with hatchability being defined as the number of eggs hatched relative to the number of fertile eggs injected.

3.2.2 Plasma Thyroxine Assay

Blood collection and storage is described in Chapter 2. Specktria radioimmunoassay kits (Coat-a-Count tubes; published specificity: 100%) were used to assess plasma thyroxine levels. The assay was first run according to the manufacturer's instructions using 20 μL of plasma and the standards provided (2, 20, 50, 100, 200, 300 nmol/L). However, under these conditions, plasma T4 levels were not detectable in most samples. Therefore, the assay was repeated using double the volumes of plasma and an extended standard curve. The standards were diluted to produce a curve that focused on lower concentrations of thyroxine (0, 2.5, 5, 10, 20, 50, 100 nmol/L). Forty microlitres of plasma were added to antibody pre-coated tubes along with the iodinated

label and incubated for two hours. The tubes were then decanted, tapped, and blotted to removed excess liquid and radioactivity measured in a Wallac gamma counter for three minutes each.

3.2.3 Maternal Transfer

Twenty four wild-type Zebra finches (*Taeniopygia guttata*) were obtained from a commercial breeder and assigned to one of four outdoor aviaries, each containing three females and three males. Birds were fed a finch seed diet with extra grit and shell provided. In addition, they were provided with three covered basket nests per aviary and a sporadic supply of grass cuttings. The birds were given two to three months to acclimatise, establish pairs and they started breeding in August 2004. Treatment was not administered until it was established that each cage had at least one breeding pair producing eggs. Once eggs were consistently produced within an aviary, all female birds within that aviary were administered a single oral dose via gavage of FIP (either 1.13×10^{-1} , 2.26×10^{-1} , or 5.65×10^{-1} mg/kg) or canola oil vehicle (OIL) at approximately the same time of day. Nests were checked daily and eggs were removed from the cage within 24 hours of laying for 14 days post-dose and immediately frozen at -20°C . The yolks were then removed at the University of Wollongong and sent on dry ice to Agrisolutions, Inc. Queensland, where they were analysed for the fipronil residues by Dr. Andrew Keats.

3.2.4 Statistical Analysis

All data were compared between trials using either Chi-square analysis (hatchability) or one-way ANOVA (body measures and plasma thyroxine level) to test

for trial effects. Where there were no statistically significant differences between trials, data were pooled and analyzed as one data set. The percent hatched for each treatment was compared with the percent hatched in the oil control group using a 2 x 2 contingency table and Chi-square analysis. Likewise, morphometric data were first checked for normality and then analyzed between treatments by one way ANOVA. Chick weights at 24 hours were regressed against egg weights at Day 0 of development and the residuals were analyzed by one way ANOVA. Lastly, plasma thyroxine levels were fitted to the standard curve (cubic spline relationship), adjusted for the volume of plasma and analyzed by one way ANOVA. All ANOVA's demonstrating statistically significant differences between variables were followed by the Tukey-Kramer's pairwise multiple comparison post hoc tests to determine which comparisons were statistically significant. The α level for all tests was set at 5% unless otherwise noted. Statview Version 5.0.1 (SAS Institute Inc, North Carolina) software was used to carry out all statistical evaluations. Values are presented as means +/- the standard error.

3.3 Results

3.3.1 Hatchability

The proportion of chicks that hatched for all treatments did not differ between trials, with the exception of the fipronil 1.13×10^{-6} mg/kg treatment group, which just reached significance ($df = 21$; $p = 0.0467$). Because of this and because of small sample sizes for some treatments, the hatchability data for both trials was combined for statistical analysis.

The CONTROL group experienced a hatchability of 75%, lower than expected for untreated eggs. The subsequent hatchability rates have been adjusted to the CONTROL group for means of comparison (Figure 3.1). Actual hatchability rates are presented in Appendix 2.

The hatchability of the FIP treated groups in general decreased as the dose increased, with the exception of the FIP 1.13×10^{-5} mg/kg exhibiting a lower hatchability than FIP 1.13×10^{-4} mg/kg (38.1% and 60.6% respectively; Figures 3.1). The lowest dose of fipronil (FIP 1.13×10^{-7} mg/kg) demonstrated the highest hatchability rate with 84.2% of eggs injected resulting in chicks and the highest dose (FIP 1.13×10^{-2} mg/kg) produced the lowest hatchability rate at a mere 11.1%.

Despite these trends, all FIP-treated groups were not statistically significantly different from OIL-treated group (Table 3.1). In fact, three of the six FIP groups (1.13×10^{-7} , 1.13×10^{-6} , and 1.13×10^{-4} mg/kg) had higher hatchability rates than the OIL (84.2%, 78.8%, 60.6%, and 57.1%, respectively). There was a statistically significant difference observed between the hatchability of OIL and CONTROL ($X^2 = 6.429$; $p = 0.0112$).

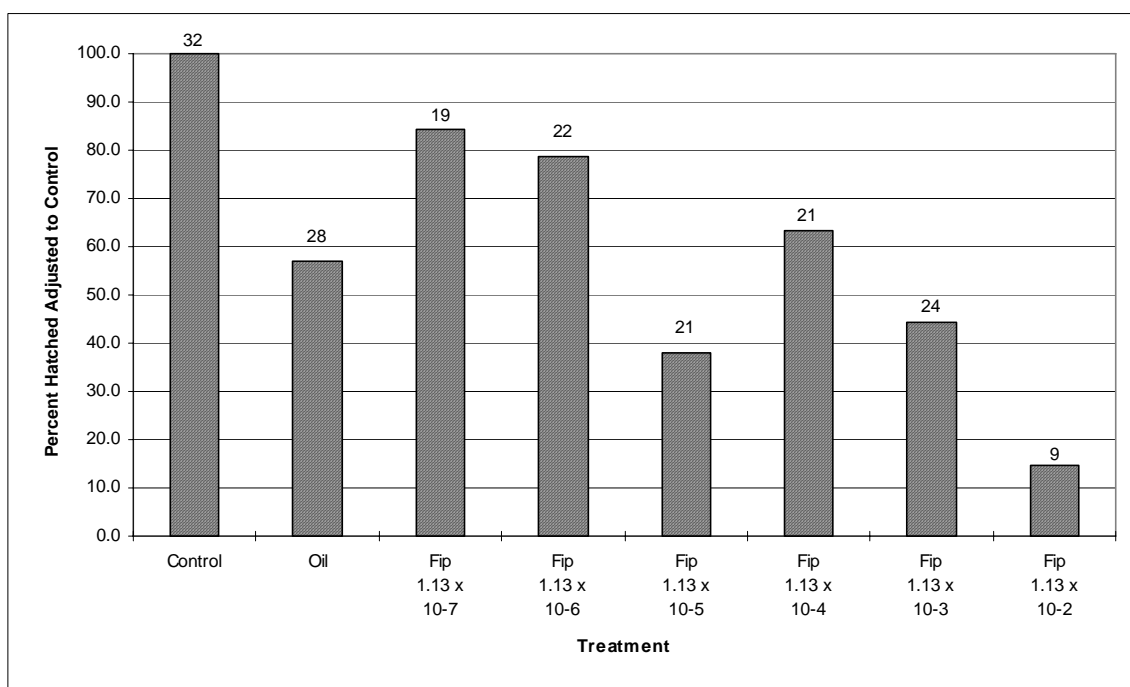


Figure 3.1: Percent hatchability (the number of eggs that hatched of all fertile eggs injected) adjusted to the hatchability of the group, which received no injection (CONTROL) in domestic chicken eggs exposed to a single dose of fipronil *in ovo*. (Numbers above bars indicate sample size).

Table 3.1 Chi-square p-values for the hatchability in domestic chicken eggs exposed to a single dose of fipronil *in ovo* for each treatment as compared to the hatchability of the OIL or CONTROL group.

Treatment (mg/kg)	X ² value vs. CONTROL	p-value vs. CONTROL	X ² value vs. OIL	p-value vs. OIL
Fip 1.13 X10 ⁻⁷	0.805	0.3696	1.867	0.1718
Fip 1.13 X10 ⁻⁶	1.530	0.2162	1.299	0.2545
Fip 1.13 X10 ⁻⁵	11.127	0.0009	1.054	0.3046
Fip 1.13 X10 ⁻⁴	4.133	0.0420	1.110	0.7402
Fip 1.13 X10 ⁻³	9.722	0.0018	0.495	0.4816
Fip 1.13 X10 ⁻²	12.049	0.0005	3.012	0.0827

3.3.2 Morphometric Comparisons

Of the eggs that did hatch, three from fipronil treated groups had to be manually assisted by breaking the shell by hand and removing the chick. Eggs numbers 3 (FIP 1.13×10^{-7}), 37 (FIP 1.13×10^{-4}) and 50 (FIP 1.13×10^{-3}) had pipped through the shell and were peeping, but remained in this state more than 24 hours. At this stage, the shell was removed and the chick was placed in the brooder cage; these chicks could not have emerged from the shell without assistance. All three had body weights much lower than the means for their respective treatment groups (32.88, 38.6 and 30.6 g, respectively). Tarsus lengths for all three were also shorter than their respective treatment means, with the tarsus of number 50 dramatically so (21.84, 21.84 and 15.26 mm, respectively). While these observations are anecdotal and not statistically significant, they do demonstrate the potential for stunted growth within the FIP treated embryos.

There were no statistically significant differences between treatments in body weight, skull length, or tarsus length twenty-four hours post-hatch when compared to OIL-treated or CONTROL eggs (Table 3.2). There was no dose-response pattern in body weights or tarsus lengths, nor was there a pattern suggested for any morphological measures. Body weight means ranged from 43.65 g (fipronil 1.13×10^{-2} mg/kg) to 48.61 g (fipronil 1.13×10^{-5} mg/kg), whereas the longest skull length (35.35 mm) was demonstrated by the oil control group and the shortest (31.30 mm) belonged to the fipronil 1.13×10^{-7} mg/kg treatment group. Tarsus lengths ranged from 21.69 mm in the fipronil 1.13×10^{-2} mg/kg treatment group to 24.18 mm in the oil group. The length of the excised femur differed significantly between the FIP 1.13×10^{-3} mg/kg treatment

and CONTROL with the fipronil treated animals displaying femurs smaller by more than 4 mm (Table 3.1). However, no other treatments were statistically distinguishable from OIL or CONTROL ($F_{1,19} = 1.863$; $df = 19$; $p < 0.1329$).

Table 3.2 Descriptive statistics for body measures taken 24 hours post-hatch in domestic chicken eggs exposed to a single dose of fipronil *in ovo*.

		Body Weight		Skull Length		Tarsus Length		Excised Femur Length	
Treatment mg/kg	N	Mean (g)	Std. Error	Mean (mm)	Std. Error	Mean (mm)	Std. Error	Mean (mm)	Std. Error
Fip 1.13 X 10^{-7}	11/4	47.10	1.27	31.30	0.35	23.20	0.31	24.0	0.223
Fip 1.13 X 10^{-6}	13/10/1	48.60	0.93	34.74	0.18	23.71	0.25	23.9	-
Fip 1.13 X 10^{-5}	5/4/3	48.61	2.94	34.58	1.25	23.79	0.62	24.1	0.640
Fip 1.13 X 10^{-4}	11/9/3	44.74	1.31	35.12	0.25	23.30	0.32	23.5	0.350
Fip 1.13 X 10^{-3}	7/3/	44.01	2.96	34.49	0.95	22.61	1.34	20.9	2.647
Fip 1.13 X 10^{-2}	2	43.65	3.06	-	-	21.69	1.11	24.6	0.820
Oil	21/20/5	46.21	0.62	35.35	0.34	24.18	0.20	23.8	0.543
Control	11/10/6	48.19	1.17	34.72	0.32	23.98	0.67	25.2	0.389
df		73		64		73		19	
P value		0.1191		0.5153		0.1782		0.1329	
F value		1.7135		0.8793		1.5076		1.863	

3.3.3 Chick Mass Vs. Egg Weight

There was a statistically significant relationship between chick mass at 24 hours post hatch and egg mass at day 0 of development ($R = 0.729$; $p < 0.0001$; Figure 3.2).

In general, masses of the FIP 1.13×10^{-7} , 1.13×10^{-4} , 1.13×10^{-3} and 1.13×10^{-2} mg/kg treatment groups fell below the regression line, while the remaining treatment groups (1.13×10^{-6} and 1.13×10^{-5} mg/kg), OIL and CONTROL were above the line, as indicated in a comparison of residuals (Figure 3.3). FIP 1.13×10^{-3} mg/kg had the largest mean residual and thus the greatest deviation from the line of regression. However, no treatment group had residual sizes that differed statistically from OIL or CONTROL ($F_{1,73} = 1.1485$; $\alpha = 0.1$; $p = 0.3430$).

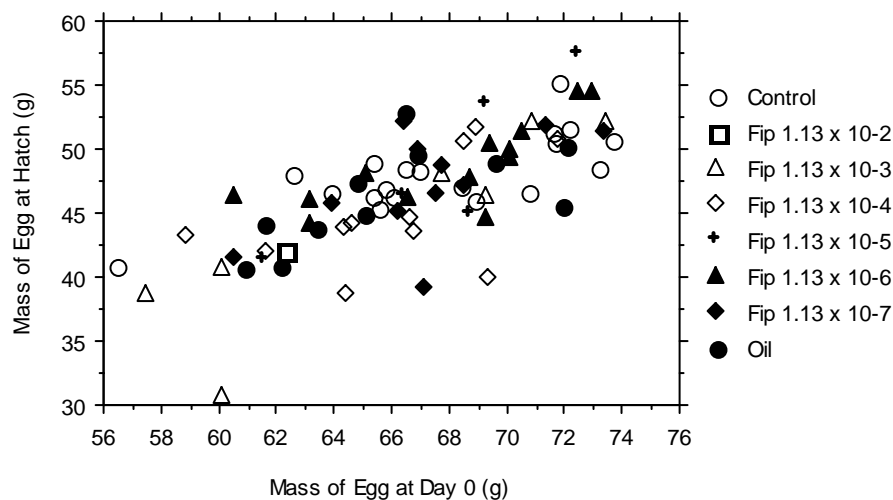


Figure 3.2 Chick mass at hatch compared to egg mass at day 0 of development in domestic chicken eggs exposed to a single dose of fipronil *in ovo*. The solid line represents the regression of hatchling mass relative to egg mass for all treatments combined.

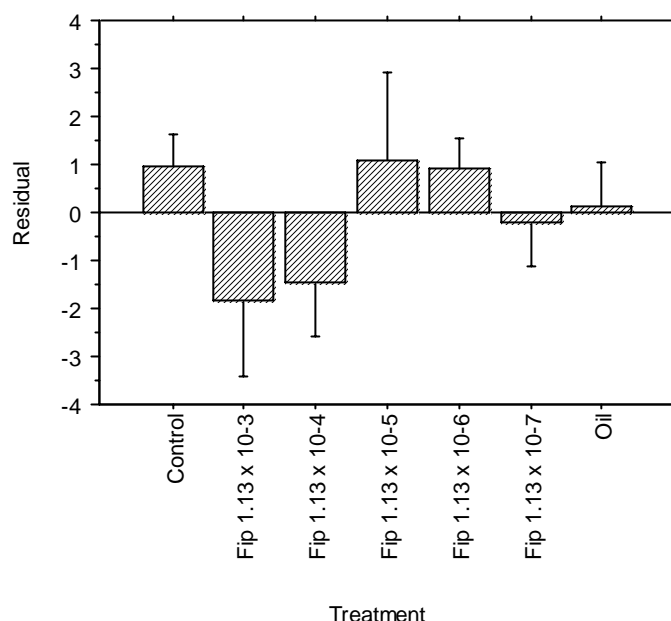


Figure 3.3 Mean residuals of actual weight at day 0 of development to the line of regression shown in Figure 2 between mass at hatch and weight of egg at day 0 for eggs of the domestic chicken treated with a single dose of fipronil *in ovo*. Error bars indicate one standard error from the mean.

3.3.4 Plasma Thyroxine Levels

In genera, thyroxine levels increased from the FIP 1.13 X10⁻⁶ mg/kg to FIP 1.13 X10⁻³ mg/kg treatments (Figure 3.4). FIP 1.13 X10⁻³ mg/kg had the highest value (6.543 nmol/mL), whereas fipronil 1.13 X10⁻⁶ mg/kg had the lowest (4.24 nmol/mL). There were no statistically significant differences between treatments and OIL in plasma thyroxine levels, however post-hoc analysis revealed a statistically significant difference between FIP 1.13 X10⁻⁶ mg/kg and FIP 1.13 X10⁻³ mg/kg ($F_{1,42} = 3.562$, $df = 42$; $p = 0.009$). Fipronil 1.13 X10⁻⁶ mg/kg is only marginally lower than OIL (0.053 nmol/L) and when analyzed with the Tukey-Kramer post hoc test with $\alpha = 1.13 \times 10^{-3}$ is statistically significantly different from oil.

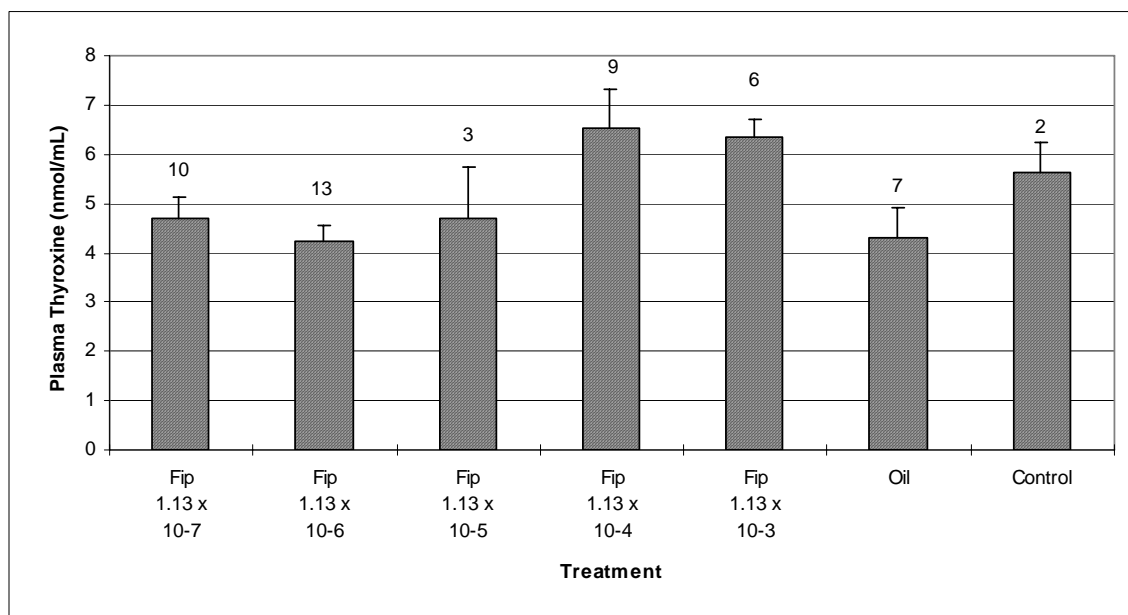


Figure 3.4 Plasma thyroxine levels (nmol/mL) in day old chicks exposed to an acute dose of fipronil *in ovo*. Bars equal one standard error from the mean; numbers above bars equal sample size.

3.3.4 Maternal Transfer

Female Zebra Finches observed for 10-30 min after dosing with fipronil did not exhibit any overt symptoms suggesting toxicity. After dosing, low levels of fipronil residues were found in 15 of the eggs laid by dosed females within 2 weeks of dosing, with median levels ranging from 0.06 to 0.5 mg/kg egg yolk, suggestive of a dose response (Figure 3.5). By far the dominant metabolite making up the combined residue level was the fipronil sulfone (Andrew Keats, pers. comm.).

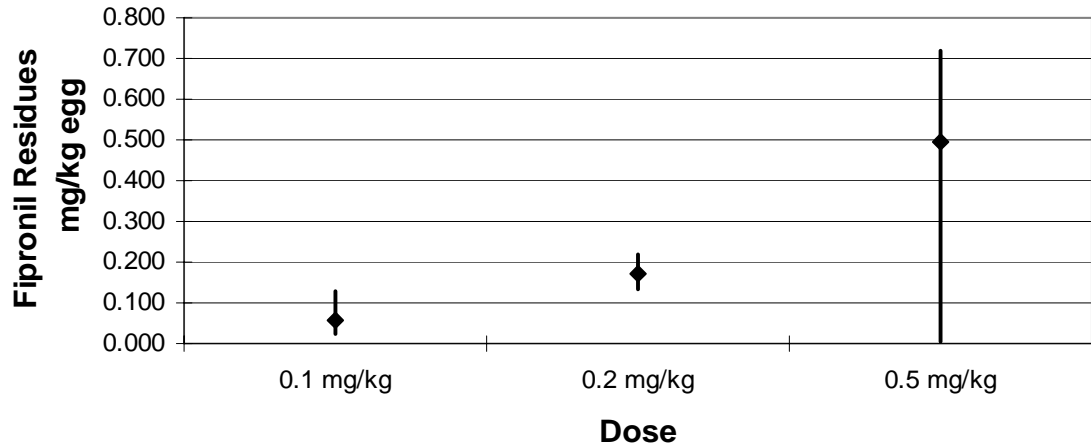


Figure 3.5 Median levels of Fipronil residues in yolk of eggs laid by dosed female Zebra Finches. Horizontal bars indicate the range of values. N = 8 for 1.13×10^{-1} mg/kg, 3 for 2.26×10^{-1} mg/kg, 3 for 5.65×10^{-1} mg/kg.

Eggs were laid sporadically and the eggs of individual females within an aviary could not be identified. Nonetheless, there does seem to be long term deposition of the fipronil in yolk (Figure 3.6), with eggs laid up to 14 days after dosing containing trace amounts. The fewest number of eggs were laid by birds given the higher doses. Laying frequency of female Zebra Finches in the four treatment aviaries varied too much prior to dosing to evaluate the effects of fipronil treatment on laying rates.

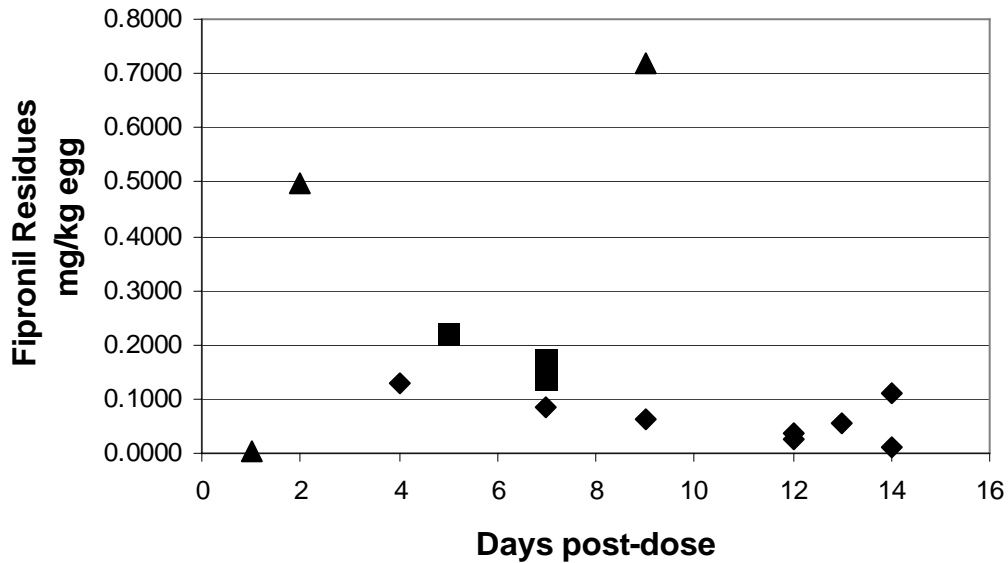


Figure 3.6 Fipronil residues in eggs laid by Fipronil-dosed Zebra Finches in relation to days after dosing. Symbols represent the dose females were given; diamonds= 1.13×10^{-1} mg/kg, squares = 2.26×10^{-1} mg/kg, triangles = 5.65×10^{-1} mg/kg dose.

3.4 Discussion

Hatchability of the CONTROL eggs was only 75%, a much lower percentage than expected. However, it is not uncommon for untreated eggs of an *in ovo* study to experience low hatchability. In a study designed to test the effects of PCB's, uninjected Leghorn chicken eggs had a reduced hatchability of 86.2% (Powell et al. 1996b). Similarly, noninjected white Leghorn chickens incubated as controls at 38°C had a 100% viability at day 4 of development, which then dramatically declined to a 21.8% hatchability (Black and Burggren 2004). In addition, the OIL treated group in the present study also experienced lower hatchability than had been expected (42.9%), also an event observed by other researchers. Leghorn chicken eggs injected on Day 0 with a control vehicle (to other toxic injections) of a mixture of lecithin, peanut oil and water

had lower than expected hatchability at 51.7% (Powell et al. 1996b). Control Japanese quail eggs that were chronically injected with saline once or three times per day experienced hatchability of 75% and 65%, respectively (Wilson and McNabb 1997). In the present study we removed infertile eggs prior to treatment, and thus these were not included in the determination of hatchability. Overall, of the 673 eggs employed in this series of experiments, 25 were infertile resulting in an infertility rate of 3.7%. This could be attributable to any number of factors such as maternal health, age or nutrition, seasonal variability in production or hatchery conditions all of which could not be controlled in this experiment, though factors that should be considered in any *in ovo* experiment.

Plasma thyroxine levels were similar to what has been previously reported for embryos near the end of incubation as well as one day old chicks. Van der Geyten and others (1997; 2002) measured thyroxine levels from day 14 of incubation to 1 day post hatch. They report plasma thyroxine levels of day old chicks at approximately 11 pmol/mL (8.5 ng/mL), with overall thyroxine levels ranging from near zero to almost 20 pmol/mL. Similarly, Engelmann et al. (2001) reported plasma thyroxine levels of day old chicks at 9 nmol/L plasma (7 ng/mL). These values are slightly higher than those obtained in the present study, which are most similar to those reported in 18 day old chick embryos present in two studies as controls (~5 nmol/mL plasma) (Kuhn et al. 1987, 1988).

Hurley, Hill and Whiting (1998) reported that adult rats demonstrated an altered thyroid state following 50 weeks of chronic treatment with fipronil and 11 weeks of recovery. Specifically, plasma levels of T4 were decreased due to an increased clearance of the hormone by the liver. In the present study, in general a higher level of thyroxine (T4) was observed as the concentration of the FIP-treated groups got higher. However, it is difficult to definitively compare the two studies, as the Hurley, Hill and Whiting review lacks data on specific dose, route of administration and age of test subjects. In addition, the effects of fipronil may differ greatly between a viviparous and oviparous animal, especially if the test subjects are at different stages of development.

Despite the weak dose effect observed in plasma thyroid hormone levels, body mass, skull length and tarsus length did not follow a similar pattern. While plasma thyroid hormone concentration has been used as a biomarker of thyroid function, previous toxicological studies have also demonstrated lack of concordance between thyroid hormone concentrations and body morphometric measures. Fence lizards exposed to cadmium *in ovo* experienced no statistically significant difference in hatchling body size or mass compared to control lizards despite the statistically significant difference in whole body T3:T4 ratio caused by an increase in T4 (Brasfield et al. 2004). The latter authors suggest that this may be due to the fact that hatchlings were sampled early in development (at hatch) rather than being allowed to develop for a period of weeks, an argument that is valid for the present study as well.

Several researchers have concluded that for pesticides that may have effects on the thyroid axis, plasma thyroid hormone levels may not be the most sensitive or appropriate measure to use as a biomarker. Aroclor 1242 (0.067 - 6.67 ppm) injected into chicken eggs at day 0, caused decreased body weight and femur length and increased plasma T4 concentrations at day 17 of development (Gould, Cooper and Scanes 1995). When this procedure was repeated on day 17 embryos treated with 0.067 or 0.67 ppm, a statistically significant increase in body weight and bone length was accompanied by a statistically significant increase in plasma T4 concentrations above controls (Gould, Cooper and Scanes 1997). Those embryos treated with 6.67 ppm had statistically significant decrease in body measures and a decrease in plasma T4 levels. In a third study, Aroclor 1242 (0.067 and 6.7 ppm) administered to chicken eggs on day 0 resulting in decreased plasma T4 levels and no effect on body weight at day 21 of development (Gould, Cooper and Scanes 1999). *In ovo* studies carried out in a different lab had similarly mixed results (Grassle and Biessmann 1982). In conclusion, while Aroclor 1242 elicits effects on the thyroid system, plasma thyroid hormone levels in relation to body measures failed to produce consistent results upon repetition of *in ovo* experiments. Likewise, further investigation may more clearly elucidate trends in the effect of fipronil on thyroid hormone levels and body measures or reveal that they are not the best indicator of an effect of fipronil on thyroid function.

The pilot trial to examine maternal transfer of fipronil into Zebra Finch eggs clearly demonstrated that fipronil can be retained in laying females for at least two weeks and be deposited in yolk. However, based on the highest fiprole concentration

measured in Zebra Finch yolk (0.7 mg/kg yolk) in a bird with an average yolk mass of 0.26 g (Lill and Fell 1990), about 2.7% of the original oral dose of fipronil (5.65×10^{-1} mg/kg female in a 12 g bird) was transferred to the yolk. It should be noted, however, that a female Zebra Finch under field conditions may be regularly consuming contaminated seed and accumulating levels of fipronil residues in fat. Thus, amounts transferred to yolk over the 2-4 days of deposition may be significantly higher than in the eggs from acutely dosed females in this study. The degree to which these levels may affect embryo development in passerine birds is unknown and should be investigated further.

Chapter 4

Fenitrothion: Hatchability, Posthatching Morphometrics and Cholinesterase Inhibition

4.1 Introduction

Fenitrothion is an organophosphate insecticide that is used to control forest and agricultural pests (Busby, White and Pearce 1991; Morin, Gaboury and Mamarbachi 1986). Fenitrothion and other OPs' principle effect and mechanism of action as an insecticide is inhibition of the enzyme acetylcholinesterase (Grue, Gilbert and Seeley 1997). Subsequently, synaptic levels of free acetylcholine rise and acetylcholinesterase activity diminishes upon exposure (Trottier et al. 1980; Kobayashi et al. 1983; Nath and Kumar 1999). The principle effect is at the neuromuscular junction where ACh accumulates, causing overstimulation of muscle fibres, including heart. Initial inhibition is rapid (Escartin and Porte 1996) and can be measured in a number of sites including the plasma, erythrocyte, and brain (Yoshida, Harada and Maita 1997). Abnormal acetylcholine activities and consequent nervous system dysfunction subsequently lead to mortality (Wright and Hillmann 1979).

Cholinesterases enzymes are found throughout the body and can be divided into two main types, acetylcholinesterases and butyrylcholinesterases. The relative amount of each as well as their respective sensitivities to contaminants, varies among species (Habig, DiGiulio, Abou-Donia 1988; Sanchez-Hernandez and Sanchez 2002; Sanchez-Hernandez 2003). Cholinesterase inhibition is often used within the toxicological

sciences as a biomarker of OP or carbamate exposure (Sanchez-Hernandez and Sanchez 2002).

The best understood function of cholinesterases is in the degradation of the neurotransmitter acetylcholine into acetyl and choline, thus removing the active neurotransmitter from the nerve synapse. This in turn allows the acetylcholine nicotinic receptor to bind to new acetylcholine and maintains the activity of the synapse. However, cholinesterases are also found in non-neural tissues, including within the blood, where their functions there are unknown. Recent investigation into the alternate functions of cholinesterases has revealed their involvement in aspects of growth and development. In humans, acetylcholinesterase acts as a stimulant for the neural release of growth hormone (Beccaria et al. 1998). In addition, acetylcholinesterase has been shown to promote nerve growth (Scholl and Sheiffle 2003) and more recently to be involved in regulating cell activity and adhesion in bone (Inkson et al. 2004).

Fenitrothion's effect on cholinesterases is not specific to insects, but, because of the ubiquitous distribution of ACh as a neurotransmitter, can be observed across taxa. Specifically, the effects of fenitrothion on birds have been well documented. Japanese quail given a single dose of 250 mg/kg of fenitrothion orally experienced tremors, salivation and convulsions within 60 minutes post-dose, and lethality was observed at higher doses (Kobayashi et al. 1983). Zebra finches ingesting a mere 1.04 mg/kg acute dose displayed a statistically significant decrease in physical activity post-dose (Holmes and Boag 1990). These outward displays of nervous system disruption may be

explained by cholinesterase inhibition. Several wild bird species observed following fenitrothion spraying had substantially depressed brain AChE activities including the Pine Siskin (*Carduelis pinus*; 76% inhibition) and the Yellow-rumped warbler (*Dendroica coronata*; 78% inhibition), which had lead to their subsequent mortality (Busby, White and Pearce 1991). Cholinesterase inhibition can be used as a biomarker for OP exposure because the level of inhibition is usually dose-dependent.

In addition to affecting the nervous system via cholinesterase inhibition, fenitrothion has also been shown to affect growth and development in avian species. Fenitrothion administered topically and via injection in eggs have reported a decrease in growth and an alteration of gait demonstrated by the curling under of toes in hatched chicks of the domestic chicken exposed to 125 mg/kg (Farage-Elawar and Francis 1987; Farage-Elawar and Francis 1988a and 1988b). White leghorn chicks experienced a decrease in hatching success and those that did hatch displayed stunted growth as well as deformed legs and gait when given 0.1 mL of a 1% solution of fenitrothion in the egg (Paul and Vadlamudi 1976). Despite fenitrothion's rapid degradation *in vivo* there is evidence that it can accumulate in fat stores (Trottier and Jankowska 1980) and be passed on to eggs during reproduction even if only in small amounts (Mihara, Misaki and Miyamoto 1979). Lastly, fenitrothion residues are detectable in egg yolk up to twelve days post exposure and the parent compound is capable of crossing the egg shell (Varga et al. 2002). Thus, the developing embryo may receive a double exposure from a fenitrothion spray event, with the compound deposited in the egg via maternal transfer as well as from external environmental residues.

Fenitrothion is capable of being transferred *in vivo* from mother to offspring in mammals, however no maternal transfer study has been conducted in avian species (Turner et al. 2002; Lehotzky et al. 1989). Previous studies have shown that other organophosphates have the ability to be passed to developing avian eggs following exposure to a breeding female, resulting in a decrease in egg and hatchling body weight (Solecki et al. 2001). While the effects of fenitrothion on chick development have been investigated, no study has introduced fenitrothion in the early stages of development. In this particular study, in an attempt to mimic a maternally transferred dose it was crucial that the pesticide be present within the egg as close to the time of laying as possible. Given fenitrothion's clearly demonstrated effect on cholinesterase as well as its direct deleterious effect on growth in ovo, we expected that chicks exposed in this study would show abnormal characteristics of growth and development.

4.2 Methods

4.2.1 Egg Treatment

All injections, egg handling, and tissue collection procedures were carried out as described in Chapter 2. Treatment solutions were made up in canola oil and were formulated so that the injection volume was between 100 or 200 μ L given an average egg weight of approximately 65 g. Eggs were treated with fenitrothion (FEN) or oil (OIL) vehicle only; there were 6 fenitrothion doses (0.001, 0.01, 0.1, 1.0, 5.0, 10.0 mg/kg egg). A control group incubated without any injection was also included (CONTROL). Fenitrothion was obtained from Sumitomo (Sumithion ULV premium

grade, 93% minimum purity, Chatswood, Australia). Hatchability was defined as the number of eggs hatched in relation to the number of fertile eggs injected.

4.2.2 Cholinesterase Activity

Because cholinesterase inhibition is often used as an indicator of OP exposure, a good deal of research has been done to establish specific cholinesterase values in individual species (Grue et al. 1991). This species specific characterisation of cholinesterase activity will allow optimization of the enzyme assay. Although chicken plasma has been previously characterized (Farage-Elawar 1991), I could not find information in the literature on plasma cholinesterase levels in day-old chicks. Because ChE characteristics change with age, particularly in young animals (Pope and Liu 1997), it was essential to characterize the plasma from chicks in this study.

Characterisation is an iterative process in which an enzymes' affinity for a substrate or enzyme inhibitor is measured based on the specific activity of the enzyme. The basic reaction whereby an enzyme reacts with a substrate, resulting in the unchanged enzyme and a new product, forms the basis of the Ellman assay. Specifically, AChE specific substrate and inhibitory properties are used to determine the specific inhibition of individual OP or carbamate pesticides.

Total cholinesterase (ChE) activity was measured for all 1-day old chicks using the method previously described by Ellman et al. (1961) and modified by Gard and Hooper (1993) for use on a 96-well spectrophotometric plate reader (Bio Tec

PowerWave X 340, Winooski, VT, USA). The spectrophotometer was set in kinetic mode and measured absorption at 412 nm for two minutes with readings taken at 15 second intervals with a zero second lag phase upon initiation of the assay (addition of substrate). Components of the standard ChE assay were 0.05 M Tris assay buffer (pH 8.0), DTNB (5,5'-dithio-bis(2-nitrobenzoic acid)), diluted enzyme, acetylthiocholine (AThCh), and iso-OMPA (tetraisopropyl pyrophosphoramidate; all reagents obtained from Sigma Chemical Co., Australia). DTNB served as a chromogen coupled to the plasma-substrate moiety, AThCh the substrate, and iso-OMPA a specific butyrylcholinesterase inhibitor used in this assay. All reagents were kept on ice until aliquoted to an individual well; the assay was run in triplicate at 25°C. The volumes of the reagents per well are outlined in Table 1; the final volume in every well was 250 μ L.

Acetylcholinesterase (AChE) was differentiated from butyrylcholinesterase (BChE) by a five minute pre-incubation with iso-OMPA prior to adding the substrate. ChE activities were converted from absorbance units/minute to umoles AThCh hydrolyzed/minute/ml plasma using the extinction coefficient, 9520 $\text{cm}^{-1}\text{M}^{-1}$ (Ellman et al. 1961). BChE activity was calculated as (Total ChE – AChE).

	Blank Standard	Total ChE Activity	AChE Activity	Final Concentration
Tris assay pH 8.0 Buffer	200 μ L	170 μ L	150 μ L	
DTNB	20 μ L	20 μ L	20 μ L	3.23×10^{-4} M
Diluted Enzyme	0 μ L	30 μ L	30 μ L	
AThCh	30 μ L	30 μ L	30 μ L	8.0×10^{-4} M

Iso-OMPA	0 μ L	0 μ L	20 μ L	1 X 10 ⁻⁴ M
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Table 4.1 Volumes of reagents used in a cholinesterase activity assay modified from Ellman et al. 1961. (ChE – cholinesterase; AChE – acetylcholinesterase; DTNB - 5,5'-dithio-bis(2-nitrobenzoic acid); AthCh – acetylthiocholine; Iso-OMPA - tetraisopropyl pyrophosphoramidate).

The first step in characterization was to determine the optimum dilution of chick plasma that results in a rate total ChE enzyme activity of 0.10 to 0.15 A/min on the spectrophotometer. Extensive use of this assay in Dr. M. Hooper's laboratory has shown that this range minimizes the variation between measurements (Hooper, pers. comm.). Plasma was diluted 5x, 10x, and 20x; this portion of the assay was run with an AThCh concentration of 10⁻⁴ M.

Having chosen the optimal sample dilution (5 X) from step one, the second step was to use this dilution to determine the optimum iso-OMPA concentration to completely inhibit BChE without inhibiting AChE. This allows separating Total enzyme activity into that produced by AChE and, by subtracting this from Total, the activity attributable to BChE. A range of iso-OMPA concentrations, from 10⁻² to 10^{-4.5} M including zero for total ChE, was run using AThCh as substrate. Log iso-OMPA concentrations were plotted against percent of total ChE and the isoOMPA concentrations where enzyme activity reached a plateau, representing the point of complete BChE inhibition, was considered the optimum iso-OMPA concentration for this assay (see Figure 4.4 in Results).

Lastly, substrate affinities were established. Plasma was assayed at AThCh concentrations of 3.2×10^{-2} to $3.2 \times 10^{-5.5}$ M with and without an iso-OMPA preincubation ($[ISO] = 1 \times 10^{-3}$). Activities for AChE and BChE (total ChE – AChE) were determined and plotted against log substrate concentration. Maximum AChE as read off the graph determined the optimum substrate concentration for the sample.

Once the optimal iso-OMPA and substrate concentrations were determined, total plasma butyrylcholinesterase and acetylcholinesterase activity levels were measured using the methods described by Ellman et al. (1961) as adapted by Fairbrother et al. (1991). Briefly, the plasma-substrate complex is attached to the DTNB chromogen and the spectrophotometer measured the rate of production in absorbance per minute at 412 nm and 25°C. BChE, total ChE, and total AChE activity were calculated as described above.

4.2.3 Statistical Analysis

All data were compared between trials using either Chi-square analysis (hatchability) or one-way ANOVA (body measures and cholinesterase activity) to test for a trial effect. Where there were no statistically significant differences between trials, data were pooled and analyzed as a single data set. The percent hatched for each treatment was compared with the percent hatched in the oil control group using a 2 x 2 contingency table and Chi-square analysis. Likewise, body weight data as well as skull and tarsus lengths were analyzed between treatments by one-way ANOVA. Linear regression was used to correlate chick weights at 24 hours with egg weights at Day 0 of

development and the residuals were analyzed by one way ANOVA. Lastly, cholinesterase activities were analysed by one way ANOVA. All ANOVA's were followed by the Tukey-Kramer's pairwise multiple comparison post hoc test to determine which comparisons were statistically significant. The α level for all tests was 5% unless otherwise noted. Statview Version 5.0.1 (SAS Institute Inc, North Carolina) software was used to carry out all tests. Means are presented +/- the standard error.

4.3 Results

4.3.1 Hatchability

The proportion of chicks hatched per treatment did not differ between trials. Thus the hatchability data for both trials was combined for statistical analysis. As previously mentioned in Chapter 3, the CONTROL group experienced a hatchability of 75%, which was lower than had been expected from untreated eggs (Figure 4.1). The subsequent hatchability rates have been adjusted to the CONTROL group for means of comparison. Actual hatchability rates are presented in Appendix 2. In addition, the OIL and CONTROL groups differed significantly from each other, as previously discussed (Chapter 3).

In general, the hatchability of the fenitrothion treated groups did not follow a linear pattern in relation to dose, and the hatchability of most doses was not different to one another or to the oil group (Figure 4.1). The FEN 1.0 mg/kg group demonstrated the highest hatchability at 69.6% and the FEN 0.1 mg/kg group produced the lowest hatchability rate at a mere 18.2%. The FEN 0.001, 0.01, 5.0, and 10.0 mg/kg dose

groups had hatchability rates that were similar (44.4%, 50.0%, 44.4%, and 51.3% respectively) to each other as well as to the OIL group (57.1%). The FEN 0.1 mg/kg treatment group was significantly lower than the OIL group ($X^2 = 5.009$; $p = 0.0252$). All other FEN treated groups did not differ significantly from OIL, but did differ significantly from CONTROL (Table 4.2).

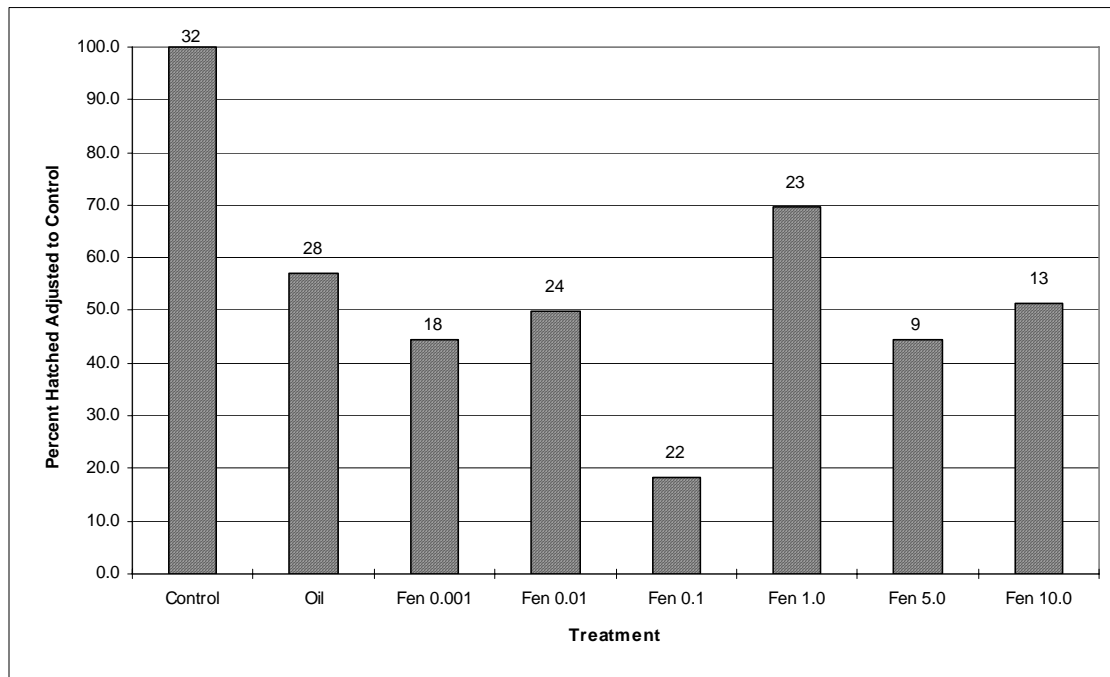


Figure 4.1 Percent hatchability (the number of eggs that hatched of all fertile eggs injected) adjusted to the hatchability of the group, which received no injection (CONTROL) in domestic chicken eggs exposed to a single dose of fenitrothion *in ovo*. (Numbers above bars indicate sample size).

Table 4.2 Chi-square p-values for the hatchability in domestic chicken eggs exposed to a single dose of fenitrothion *in ovo* for each treatment as compared to the hatchability of the OIL or CONTROL group.

Treatment	X² value vs. CONTROL	p-value vs. CONTROL	X² value vs. OIL	p-value vs. OIL
Fenitrothion 0.001	8.333	0.0039	0.417	0.5183
Fenitrothion 0.01	7.968	0.0048	0.154	0.6947
Fenitrothion 0.1	19.636	<0.0001	5.009	0.0252
Fenitrothion 1.0	3.084	0.0791	0.440	0.5071
Fenitrothion 5.0	5.423	0.0199	0.256	0.6127
Fenitrothion 10.0	5.386	0.0203	0.071	0.7904

4.3.2 Morphometric Comparisons

There was no dose-response pattern in chick mass or body morphological measures, with measurements similar across treatments. Mean body mass ranged from 45.15 (FEN 5.0 mg/kg) to 50.22 (FEN 0.1 mg/kg), whereas the longest skull length (36.07 mm) was demonstrated by the FEN 10.0 mg/kg group and the shortest (34.10 mm) in the FEN 5.0 mg/kg treatment group. Tarsus lengths ranged from 23.213 mm in the FEN 1.0 mg/kg group to 24.480 mm in the FEN 5.0 mg/kg treatment group. There were no statistically significant differences between treatments in body mass, tarsus length, skull length, or excised femur length twenty-four hours post-hatch when compared to OIL or CONTROL (Table 4.3). In general the number of excised femur samples collected for the FEN treatment groups were too small to conduct statistical comparisons, but did not differ greatly across treatments (Appendix II).

Table 4.3 Descriptive statistics for body measures taken 24 hours post-hatch in domestic chicken eggs exposed to a single dose of fenitrothion *in ovo*. Analysis was conducted using a one-way ANOVA and the Tukey-Kramer post-hoc analysis between treatments ($\alpha = 0.1$).

		Body Mass		Skull Length		Tarsus Length	
Treatment	N	Mean (g)	Std. Error	Mean (mm)	Std. Error	Mean (mm)	Std. Error
Fenitrothion 0.001	5	47.43	1.97	34.81	0.39	23.44	0.42
Fenitrothion 0.01	9/8	48.87	2.13	34.78	0.25	23.71	0.35
Fenitrothion 0.1	2	50.22	0.58	35.87	0.13	23.29	0.01
Fenitrothion 1.0	11	45.72	1.70	35.07	0.35	23.21	0.40
Fenitrothion 5.0	2	45.15	2.15	34.10	0.04	24.48	1.42
Fenitrothion 10.0	5	46.51	0.94	36.07	0.56	23.62	0.38
Oil	11	46.21	0.62	35.35	0.34	24.18	0.20
Control	21	48.19	1.17	34.72	0.32	23.98	0.67
df		58		55		58	
P value		0.5540		0.3290		0.1791	
F value		0.846		1.181		1.519	

4.3.3 Chick Mass vs. Egg Weight

There was a statistically significant relationship between chick and egg masses ($r^2 = 0.554$); (Figure 4.2). In general, means of the residuals for the FEN 1.0, 5.0, 10.0 and the oil control group fell below the regression line, while the remaining lower dose groups and CONTROL were above (Figure 4.3). FEN 1.0 mg/kg had the largest mean residual and thus, the greatest deviation from the line of regression.

However, no treatment group had residual sizes that differed statistically from OIL or CONTROL ($F_{1,59} = 2.305$; $df = 59$; $p = 0.038$). The FEN 0.01 and 1.0 mg/kg treated groups differed significantly, with the mean residual size of the FEN 0.01 mg/kg

treated group larger than that of the FEN 1.0 mg/kg treated group by 4.436 g. The FEN 1.0 mg/kg treated group approaches significance when compared to CONTROL, with a difference in means of -3.022 (-2.283 FEN 1.0 mg/kg; 0.739 CONTROL; $\alpha = 0.1$).

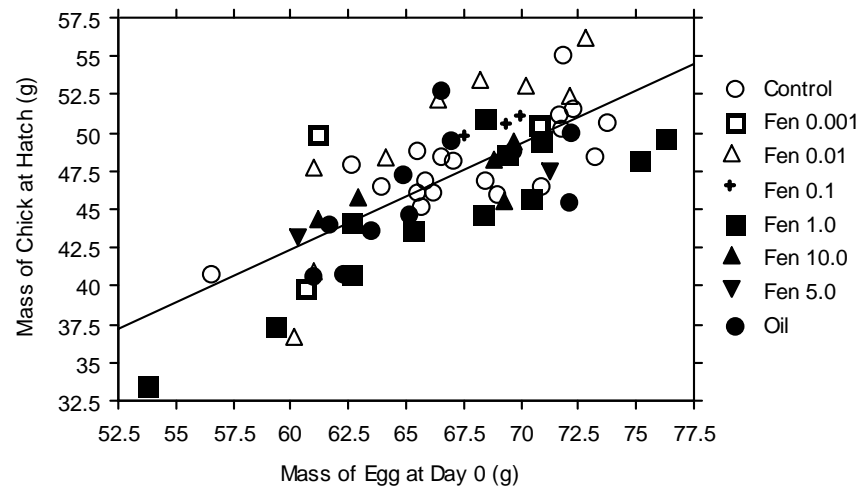


Figure 4.2 Chick mass at hatch regressed against egg mass at day 0 of development in domestic chicken eggs exposed to a single dose of fenitrothion *in ovo*.

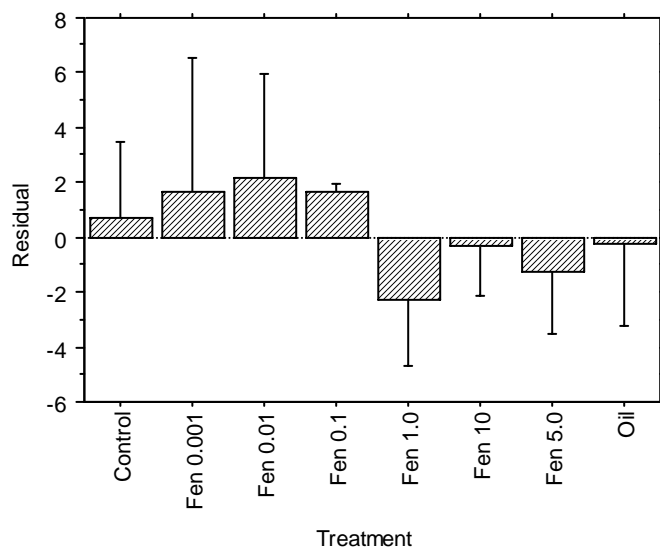


Figure 4.3 Mean residuals of egg mass at day 0 to the line of regression between mass at hatch and mass of egg at day 0 for eggs of the domestic chicken treated with a single dose of fenitrothion *in ovo*. Error bars indicate one standard error from the mean.

4.3.4 Cholinesterase Characterisation

First, chick plasma samples were run at different dilutions to determine the appropriate dilution for further characterization. From dilutions of 5x, 10x and 20x, the 5x dilution produced slopes closest to 170 and, thus, was used for the rest of the assay. Secondly, the optimal iso-OMPA concentration was determined. ChE activity plateaus around $10^{-2.55}$ M, indicating the optimal iso-OMPA concentration for complete BChE inhibition (Figure 4.4). Lastly, substrate affinities were established using activities for AChE and BChE (total ChE – AChE) plotted against log substrate concentration. The midpoint of the sigmoidal AChE curve is considered to be the optimal substrate concentration, which in the present study was 10^{-4} (Figure 4.5).

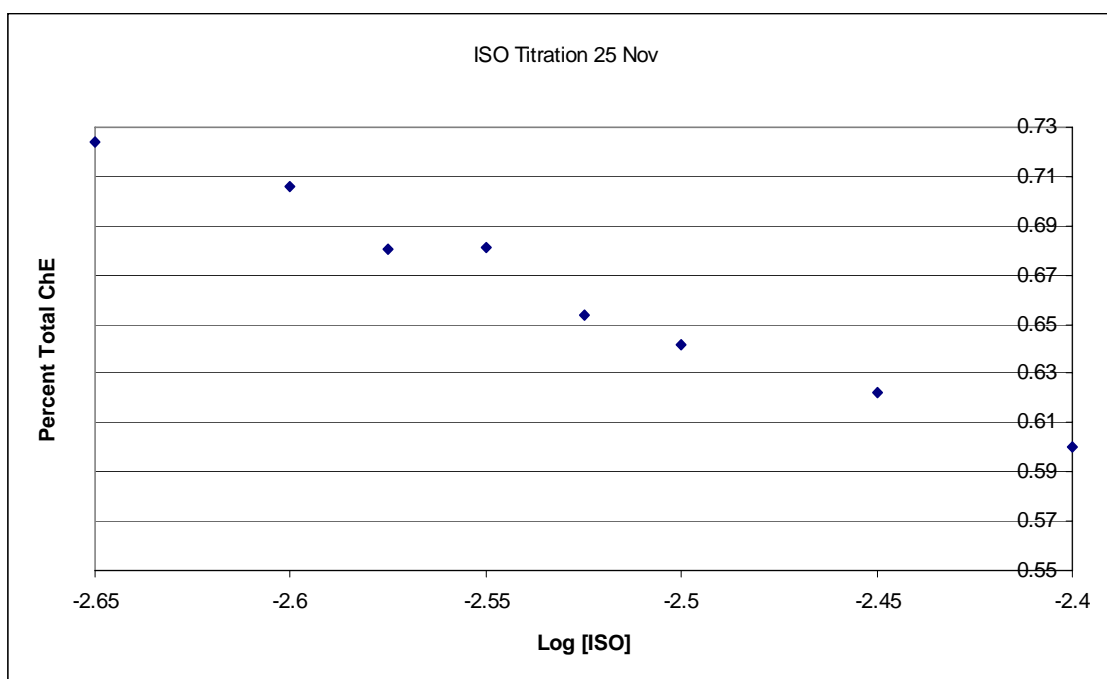


Figure 4.4 Plasma cholinesterase activity at different iso-OMPA concentrations in the domestic chicken 24 hours post hatch.

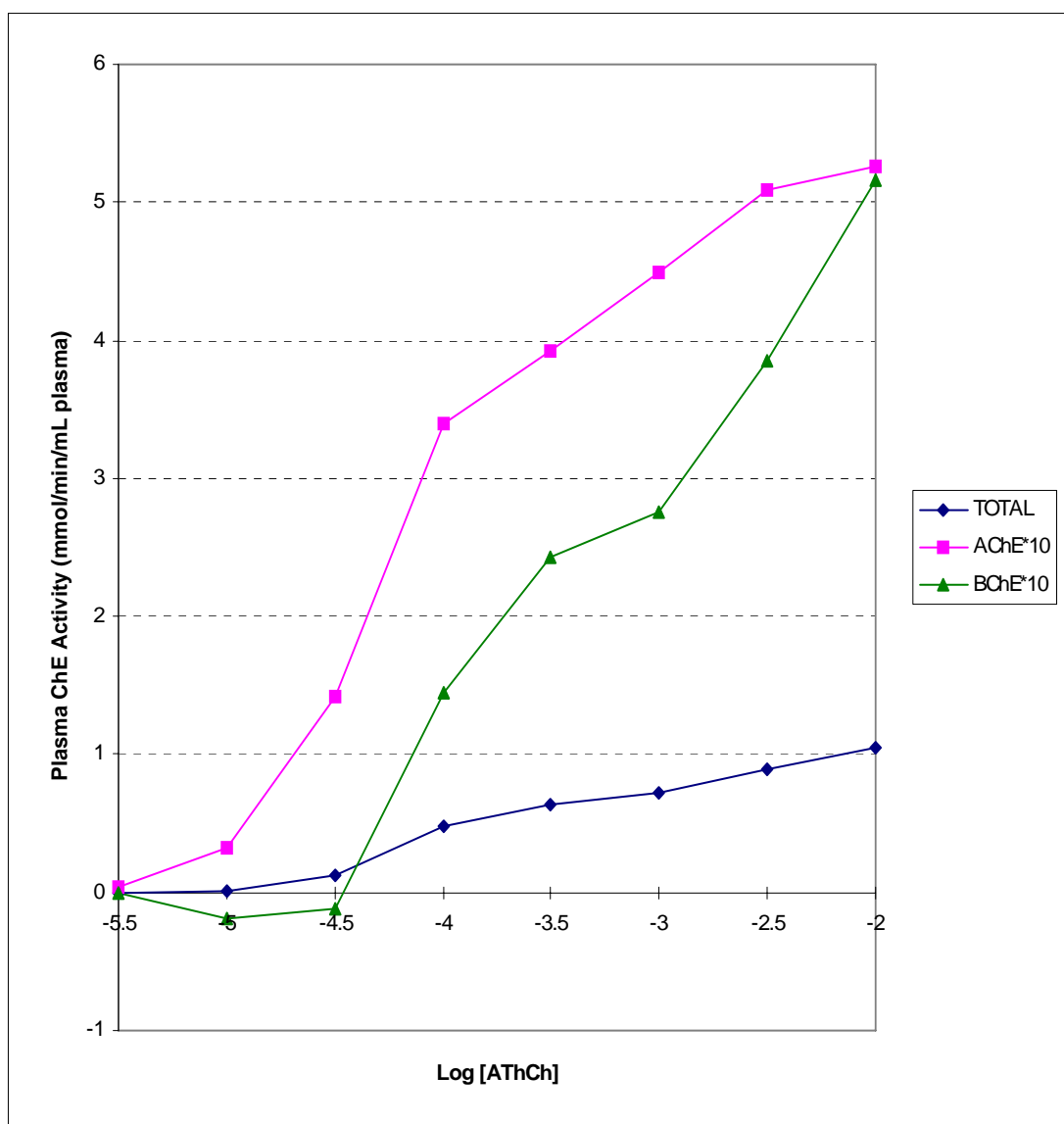


Figure 4.5 Plasma cholinesterase activity at different substrate concentrations in the domestic chicken 24 hours post hatch. A [AThCh] of 10^{-4} M was chosen for the subsequent assay.

4.3.5 Cholinesterase Inhibition

Plasma total ChE activities followed a generally linear pattern with the highest activity in the FEN 1.0 group and the lowest in the FEN 0.0001 group (Figure 4.6). The FEN 10.0 group breaks this pattern with a total ChE activity lower than that of the FEN

1.0 group. The total ChE activity of the OIL group was lower than both FEN 1.0 mg/kg group by 0.12 $\mu\text{mol}/\text{min}/\text{mL}$ plasma. Mean AChE activities also followed this pattern except that the mean AChE activity of OIL was lower than 3 of the 5 FEN treatment groups. Mean AChE activities ranged from 0.341 $\mu\text{mol}/\text{min}/\text{ml}$ in the FEN 0.1 mg/kg group to 0.516 $\mu\text{mol}/\text{min}/\text{ml}$ in the FEN 1.0 mg/kg group. BChE activities followed no observable pattern.

Cholinesterase activity readings were not normally distributed and therefore were sine-transformed. The total ChE activity of the FEN 0.0001 mg/kg group was statistically different from FEN 1.0 mg/kg (Table 4.4). No other ChE activities differed statistically from the activities of the OIL group or from other treatments when analysed via the Tukey-Kramer test with an α of 0.05. When α is set at 0.1, however, the BChE activity of FEN 1.0 mg/kg and OIL both become significantly different to the BChE activity of FEN 0.001 mg/kg.

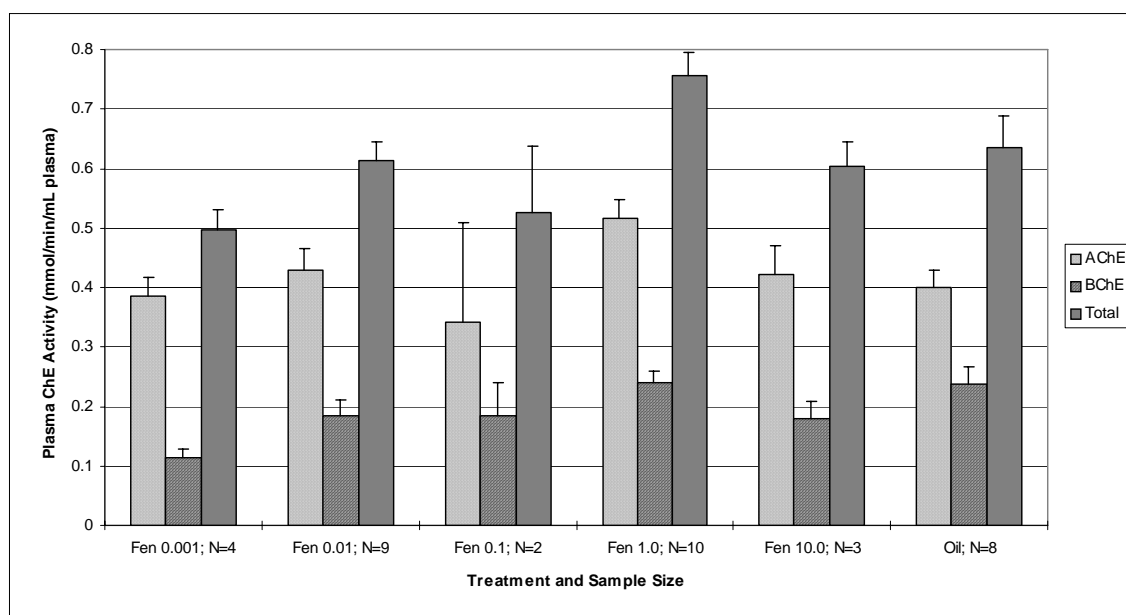


Figure 4.6 Mean cholinesterase plasma activity after *in ovo* exposure to fenitrothion or canola oil in eggs of the domestic chicken. Error bars indicate the standard error.

Table 4.4 Descriptive statistics for ChE activity 24 hours post-hatch in domestic chicken eggs exposed to a single dose of fenitrothion or oil *in ovo*.

	df	F value	p value
Total ChE	32	3.1709	0.0148
AChE	32	1.9227	0.1073
BChE	32	2.1505	0.0745

4.4 Discussion

The results of this study differ from those of other *in ovo* experiments involving fenitrothion. In a previous study, fenitrothion (125 mg/kg) injected into the eggs of chickens induced statistically significant inhibition of acetylcholinesterase for sixteen days post-dose (Farage-Elawar and Francis 1988). However, the dose used by Farage-Elawar and was more than ten-fold higher than the highest fenitrothion dose used in the

present study. In addition, Farage-Elawar and Francis injected the pesticide on day 15 of development. Further, they examined ChE inhibition at nineteen days post treatment (or 13 days post hatch). At this time, ChE inhibition was less than 3% and by 23 days post injection (17 days post hatch) recorded zero inhibition.

The doses used in this study may have been too low to elicit an effect on the developing embryo, either because of species' tolerance to the compound or due to rapid degradation or metabolism of the compound. Bearded dragons dosed with two concentrations of fenitrothion exhibited marked plasma ChE inhibition at the higher dose (20 mg/kg), but very little inhibition at the low dose (2 mg/kg; Bain et al 2004). In addition, Varga et al. (2002) measured fenitrothion residues from Day 1 to Day 12 of incubation from chicken eggs that were dosed on Day 0. Each egg had received 0.15 mg of fenitrothion, resulting in measures of 333 ng/g of egg on Day 1, but by Day 6 fenitrothion levels had decreased to less than 5 ng/g and by Day 12 were completely undetectable. In the present study, the highest doses were similar to those in the Varga study (10 mg/kg fenitrothion in a 65 g egg is approximately 0.65 mg per egg), while the lowest dose was four orders of magnitude less (or 0.000065 mg per egg). Thus, in the present study with chick plasma assayed for ChE inhibition 19 days post dose, any effects may have been eliminated due to the rapid metabolism of fenitrothion.

In addition, some vertebrates exposed to fenitrothion have shown an ability to recover from exposure and subsequent ChE inhibition (Fleming and Bradbury 1983; Escartin and Porte 1996). In the present study, an inhibition of ChE may have occurred

during development within days of the injection, but were not demonstrable at the time of hatching. Based on Farage-Elawar and Francis (1998a) observed 3% inhibition at 19 days post injection, we might have expected to see similar inhibition in the present study at 19 days post injection. However, the higher dose in the previous study, coupled with differences in developmental stages of exposure may explain our lack of observed ChE inhibition.

In the present study there were no statistically significant differences between treatment groups on body morphometrics. Similar to this study, fenitrothion (125 mg/kg) injected into eggs of developing chickens elicited no effect on weight or weight gain of chicks post-hatch when compared to controls (Farage-Elawar and Francis 1988).

Chapter 5

The Effect of FIP or FEN Exposure on Oxygen Consumption and Metabolic Rate

5.1 Introduction

As the developing chicken embryo begins to build body tissues, an increasing amount of oxygen will be consumed and carbon dioxide produced as a greater amount of tissue becomes metabolically active. Consequently, measurements of embryonic metabolic rate correlate strongly with its stage of development. The pattern of oxygen consumption in the eggs of precocial species has been well documented. In general, metabolic rate rises steadily starting at approximately 20% through incubation period until just prior to internal pipping of the inner shell membrane into the air cell. At this point there is a slight plateau, or in some cases a decrease, before hatching in contrast to altricial species which rise exponentially until hatch (Vleck and Vleck 1987). The precocial drop off in oxygen consumption is related to and perhaps results from a decrease in growth rate towards the end of incubation (Vleck, Hoyt and Vleck 1979), thus factors that affect growth rate may also affect metabolic processes and oxygen consumption.

As previously discussed (Chapter 3), fipronil has possible effects on thyroid hormone status. In addition to regulating growth and development, thyroid function is also strongly correlated with metabolism and processes associated with it. In the Tasmanian bettong (*Bettongia gaimardi*) injections of T4 administered to 12 week old

pouch young were associated with elevated oxygen consumption (Rose and Kuswanti 2004). In avian species, T3 directly regulates malic enzyme activity, a molecular cascade responsible for lipogenesis. Avian thyroid hormones are also known to influence thermoregulation, with chemically induced hypothyroid or hyperthyroid birds demonstrating depressed or elevated metabolic rate respectively (McNabb 1995 and citations within). Similarly, Ries and Pokrajac (1987) measured oxygen consumption and serum T3 concentrations in 4 month old female Wistar rats that were either hypothyroid, hyperthyroid or normal. Not only did they demonstrate a strong correlation between T3 levels and metabolism, the hypothyroid rats had lower values for both measures while the hyperthyroid rats had higher values for both measures when compared to the controls. Young broiler chicks kept at 20°C for seven days displayed significantly higher heat production as well as circulating T3 levels than those kept at thermoneutral temperatures (Collin et al. 2003). In terms of ontogenetic development of thermoregulation, Olson and others (1999) correlated thyroid development in red-winged blackbirds with endothermic regulation during the nestling period.

Organophosphate (OP) pesticides have also been shown to affect mammalian thermoregulatory processes by interfering with associated cholinergic pathways of the nervous system. These interruptions in normal neural function often result in an inability to maintain normal body temperature or an increased sensitivity to contaminants at non-thermoneutral temperatures (Rattner, Becker and Nakatsugawa 1987). Sprague-Dawley rats orally given 15 mg/kg chlorpyrifos experienced hypothermia followed by a fever one day post-dose, a response that is commonly seen

in laboratory mammals (Gordon 1994; Rowsey, Metzger and Gordon 2001). However, some species exposed to various doses of anticholinesterase agents have experienced hyperthermia and elevated body temperature (Gordon 1994). Despite research involving the effects of OP's on body temperature, surprisingly little investigation has been done regarding the direct effects of OP's on metabolic rate. Various fish and shrimp species demonstrated no change in oxygen uptake when exposed to the OP azinphosmethyl (Cochran and Burnett 1996) and the fat-tailed dunnart *Sminthopsis crassicaudata* endured no change in peak metabolic rate while running when exposed to freezing temperatures after ingesting 30 mg/kg fenitrothion (Buttemer, personal communication).

Given the known effects of both thyroid hormones and cholinergic pathways on thermoregulation, a disruption in normal thyroid or cholinesterase functions may perturb standard metabolic rate as well. While previous studies have investigated the concurrent effect of toxicants on thyroid status or cholinesterase inhibition and metabolic rate, none have done so in an avian species (Tomasi, Ashcraft and Britzke 2001; Bain et al. 2004).

5.2 Methods

5.2.1 Equipment

The eggs and metabolic chambers were placed in a temperature-controlled incubator that maintained the temperature at 37.5°C ($\pm 0.5^\circ\text{C}$). The methods of closed-system respirometry follow those of Vleck et al. (1980) except that reference oxygen

readings were taken from egg-free chambers to permit interpolation of initial O₂ content. The oxygen content of air samples was calculated using equation number 9 of Vleck (1987). After correcting for vapour pressure effects, CO₂ and water were removed from the air sample using soda lime and Drierite columns, respectively and oxygen content was measured using a Sable Systems oxygen analyzer (Model FC1, Henderson, NV, USA).

5.2.2 Procedure

Eggs were handled and treated as described in Chapter 2. Metabolic measurements were taken starting at day 10 of development (7 days post-dose) and then on days 12, 14, 16, 17 and 18. There were a total of eighteen metabolic chambers used in this study and no egg was measured in the same chamber twice throughout the experiment. The eggs remained in the chambers long enough so that the oxygen content of the chamber fell below 20.7% but always above 17%. Eggs that registered no oxygen consumption were considered dead and all data for those embryos were not included in subsequent analysis.

5.2.3 Data Analysis

Oxygen consumption data were analysed by a repeated measures one way ANOVA followed by Tukey's pairwise multiple comparison post hoc test to determine which comparisons were statistically significant. Oxygen consumption was regressed against both total and acetylcholinesterase activities and analysed using one-way ANOVA. Unless otherwise noted, α was set to 0.05. Statview Version 5.0.1 (SAS

Institute Inc, North Carolina) software was used to carry out all tests. Values are reported with means +/- the standard error.

5.3 Results

5.3.1 Fenitrothion

In general, from day 14 of incubation through day 18, oxygen consumption rates of FEN treated eggs were similar or slightly lower than that of the OIL treated group (Table 1). The values for the CONTROL group were slightly higher and differed significantly from the FEN 0.1 mg/kg and FEN 5.0 mg/kg treated groups ($F_{2,7} = 3.987$; $df = 7$; $p = 0.0008$). The FEN 5.0 mg/kg treated group also differed significantly from the FEN 10.0 mg/kg group, having consistently lower oxygen consumption rates.

Table 5.1 Mean oxygen consumption (mL/minute) over time of eggs of the domestic chicken given an acute dose of fenitrothion or oil vehicle *in ovo* or those that received no injection (CONTROL). N's are the number of eggs measured per treatment.

Treatment (mg/kg)	N	Day 10 (mL/min)	Day 12 (mL/min)	Day 14 (mL/min)	Day 17 (mL/min)	Day 18 (mL/min)
Fenitrothion 0.001	10	0.07 ± 0.003	0.14 ± 0.009	0.28 ± 0.012	0.37 ± 0.017	0.39 ± 0.015
Fenitrothion 0.01	14	0.04 ± 0.002	0.14 ± 0.006	0.28 ± 0.010	0.39 ± 0.013	0.39 ± 0.013
Fenitrothion 0.1	9	0.07 ± 0.003	0.14 ± 0.006	0.27 ± 0.012	0.38 ± 0.035	0.35 ± 0.031
Fenitrothion 1.0	13	0.07 ± 0.002	0.15 ± 0.009	0.28 ± 0.009	0.38 ± 0.025	0.40 ± 0.020
Fenitrothion 5.0	5	0.06 ± 0.005	0.11 ± 0.013	0.24 ± 0.011	0.34 ± 0.025	0.31 ± 0.037
Fenitrothion 10.0	7	0.07 ± 0.002	0.17 ± 0.004	0.31 ± 0.010	0.39 ± 0.034	0.42 ± 0.018
Oil	14	0.07 ± 0.002	0.15 ± 0.007	0.28 ± 0.008	0.39 ± 0.011	0.40 ± 0.013
Control	22	0.07 ± 0.009	0.15 ± 0.006	0.29 ± 0.008	0.45 ± 0.009	0.44 ± 0.008

5.3.2 Fipronil

Although the CONTROL group had oxygen consumption values consistently higher than the other treatment groups, there was no statistically significant difference between any of the controls or fipronil treated groups (Table 2; $F_{2,7} = 1.125$; $df = 7$; $p = 0.3545$).

Table 5.2 Mean oxygen consumption (mL/min) over time of eggs of the domestic chicken given an acute dose of fipronil or oil vehicle *in ovo* or those that received no injection (CONTROL). N's are the number of eggs measured per treatment.

Treatment (mg/kg)	N	Day 10 (mL/min)	Day 12 (mL/min)	Day 14 (mL/min)	Day 17 (mL/min)	Day 18 (mL/min)
Fip 1.13×10^{-2}	13	0.07 ± 0.002	0.15 ± 0.005	0.29 ± 0.005	0.40 ± 0.013	0.40 ± 0.015
Fip 1.13×10^{-3}	16	0.07 ± 0.003	0.15 ± 0.006	0.28 ± 0.010	0.41 ± 0.014	0.42 ± 0.014
Fip 1.13×10^{-4}	8	0.07 ± 0.003	0.14 ± 0.006	0.28 ± 0.017	0.39 ± 0.029	0.42 ± 0.023
Fip 1.13×10^{-5}	13	0.07 ± 0.002	0.16 ± 0.005	0.27 ± 0.009	0.41 ± 0.021	0.40 ± 0.019
Fip 1.13×10^{-6}	10	0.07 ± 0.003	0.15 ± 0.006	0.28 ± 0.013	0.38 ± 0.024	0.38 ± 0.030
Fip 1.13×10^{-7}	1	0.07	0.17	0.26	0.38	0.40
Oil	14	0.07 ± 0.002	0.15 ± 0.007	0.28 ± 0.008	0.39 ± 0.011	0.40 ± 0.013
Control	22	0.07 ± 0.009	0.15 ± 0.006	0.29 ± 0.008	0.45 ± 0.009	0.44 ± 0.008

5.4 Discussion

Oxygen consumption values obtained in the present study are similar to those reported previously for chicken embryos (Appendix I). Rahn, Paganelli and Ar (1974)

recorded oxygen uptake rates of chicken embryos throughout incubation. They reported rates at day 15 of approximately 500 mL/day (0.347 mL/min) increasing at day 19 to nearly 600 mL/day (0.417 mL/min). Vleck and Vleck (1987) reported an oxygen consumption rate of 570 mL/day (0.396 mL/min) in chicks just prior to pipping. While this timepoint lies just outside the present study's measurements (day 19 or 20), I recorded similar values towards the end of incubation (day 18).

Ries and Pokrajac (1987) demonstrated a high correlation between serum T3 levels and metabolic rate in juvenile female Wistar rats. Those that were hypothyroid had lower oxygen consumption rates as well as lower serum T3 concentrations than rats with normal thyroids. Given fipronil's reported effects on the thyroid (Chapter 3) one might expect it to produce a decreased metabolic rate in chicken embryos if it similarly affected their T4 secretion. In the present study, while not statistically significant, the FIP treated animals experienced oxygen consumption rates lower than those of the CONTROL group across incubation, indicating that there may be a depression of metabolic rate due to treatment. The OIL treated group, however, displayed oxygen consumption rates similar to those of the FIP treated groups, which was unexpected. Because the OIL treated group also exhibited an unexpectedly low hatchability rate, there may be factors affecting all embryos exposed to oil that we cannot fully account for, despite our efforts to eliminate this problem in our methods development (Chapters 2 and 3).

In a previous study investigating the effect of fenitrothion on cholinesterase inhibition and standard metabolic rate, bearded dragons (*Pogona viticeps*) were exposed to an acute dose of either 2 or 20 mg/kg by ingestion (Bain et al. 2004). In the 20 mg/kg group, total cholinesterase, acetylcholinesterase and butyrylcholinesterase were all significantly depressed compared to controls, however there was no statistically significant difference in standard metabolic rate between treatments. In the present study, while not significantly different from the OIL treated group, the FEN treated groups experienced a decrease in total cholinesterase and acetylcholinesterase activity as the dose of fenitrothion increased (Chapter 4). This indicates that while fenitrothion may have affected cholinergic pathways, there was not enough interference to elicit an effect on standard metabolic rate. The hypotheses that an insufficient dose of fenitrothion was administered or that the embryo was able to recover are discussed in Chapter 4.

When oxygen consumption of FEN treated embryos were compared with both total and acetylcholinesterase activities, there was no statistically significant relationship (Total: $R = 0.043$; $df = 36$; $p = 0.80$; AChE: $R = 0.22$; $df = 36$; $p = 0.19$). This indicates that the metabolic rate of day 18 embryos was not influenced by either total or acetylcholinesterase activity. In contrast, when oxygen consumption of FIP treated embryos were compared to plasma T4 concentrations, there was a statistically significant relationship ($R = 0.31$; $df = 36$; $p = 0.03$). Therefore, although FIP treatment suggested an inverse relationship between dose and plasma T4 levels, this relationship did not significantly affect oxygen consumption.

Chapter 6

Conclusions and Future Directions

6.1 Conclusions

The aims of this research were to investigate the effects of an *in ovo* injection of fipronil or fenitrothion on the development of the domestic chicken to mimic the pesticide being transferred into egg yolk from an exposed mother. Outcome measurements included hatchability, body growth and morphometrics, and embryonic oxygen consumption for embryos exposed to both pesticides. In addition, given fipronil's reported effects on thyroid status and fenitrothion's known inhibition of cholinesterases, measurements were taken to assess these parameters in their respective treatment groups.

Overall the effects of fipronil on the growth and development of chicken embryos demonstrated some linear trends in relation to dose, though none of these were statistically significant. Hatchability followed a linear pattern, with the lowest dose (FIP 1.13×10^{-7} mg/kg) resulting in the largest proportion hatched and the highest dose (FIP 1.13×10^{-2} mg/kg) with the lowest proportion. Likewise, plasma thyroxine levels also followed a linear model with chicks given the higher fipronil doses having higher levels of the hormone and the lower doses having less. Conversely, there was no pattern in body mass or skeletal measures. While oxygen consumption rates tended to be lower in FIP dosed eggs than the CONTROL group, there was no discernable influence of FIP on embryonic metabolic rate.

In relation to hatchability, body growth, and development, treatment with fenitrothion yielded no statistically significant differences between treatments nor suggested any possible trends across groups. While the inhibition of total cholinesterase and acetylcholinesterase followed a relatively linear pattern in relation to dose, there were no statistically significant differences between FEN treated groups and the OIL treated group in these measurements. Butyrylcholinesterase activity did not differ significantly between any treatments and suggested no identifiable pattern. Oxygen consumption rates for the FEN treated groups were lower overall than the CONTROL group and for the FEN 0.1 mg/kg and FEN 5.0 mg/kg treated groups this difference was statistically significant. However, no FEN treated group had metabolic rates that differed significantly from those of the OIL treated group.

In Chapter 5, I compared the plasma T4 concentrations of chicks from fipronil treated eggs with their oxygen consumption at day 18 of development. A strong positive relationship between oxygen consumption and circulating thyroid hormone levels has been previously demonstrated (Ries and Pokrajac 1987) and, thus, was also expected in the present study. A linear regression between these two parameters did in fact reveal this pattern across treatment groups. Although there was a statistically significant relationship between T4 levels after hatching and VO₂ at day 18, FIP did not cause a clear enough distinction in T4 levels (Chapter 3) to result in a statistically significant effect on metabolic rate. However, larger sample sizes may be needed to identify small differences.

Would the effects on the individual parameters of body and skeletal growth been clearer if the study was conducted at a larger scale? The power analysis for body morphometrics, plasma thyroxine levels and cholinesterase inhibition indicate that while larger sample sizes would be required to overcome the extent of variance, the numbers used in the present study are reasonable. For example, the number of samples predicted in order to show a statistically significant difference in body mass for the fipronil treated groups is 100, whereas the number actually analysed in the present study was 81. Likewise, the number of samples needed to show statistically significant differences between acetylcholinesterase inhibition might be expected to be 48 compared to the 37 available in this study. In addition, the presence of chicks that required manual assistance to hatch and their specific measurements are of importance. Three of the four chicks were from fipronil treated groups and displayed extremely low mass and tarsal length, and oxygen consumption rates in a dose dependent manner. In addition, there were two other FEN treated chicks (0.01 and 0.1 mg/kg) that pipped through the shell and died shortly thereafter. With a larger sample size, the frequency of these failed hatchings may have increased and the reasons for their demise further elucidated.

Another improvement in the study design would be to make the injection protocols even more uniform. Careful effort was taken when injecting the test substances into the egg so that the injection avoided disruption of the embryo and was delivered as uniformly as possible to each egg. Despite this, it is possible that slight differences in egg orientation or injection angle placed the test substance at dissimilar

locations within the yolk of each individual egg. It has been previously suggested that developing eggs may utilize yolk lipids selectively and asymmetrically during development (Grau, 1974; Astheimer, pers. comm). When injecting into the yolk, we made the assumption that fipronil and fenitrothion would disperse throughout the yolk. However, we do not know the rate of dispersal or its degree of uniformity in distribution, and thus embryos may have been exposed to different levels of pesticide at varying times, depending on the injection's proximity to the embryo or its vasculature. For future studies, it would be beneficial to devise a standardized injection method targeting egg and needle orientation so that the site of pesticide deposition is the same in each yolk relative to the egg's size and shape.

6.2 Future Directions

While we now have a better understanding of the developmental effects of fipronil and fenitrothion in an oviparous organism, there are still many questions left to investigate. In addition to the possible alterations to the present study design mentioned above, there are also related experiments that may provide a greater insight into the potential teratogenic or other physiological effects of fipronil and fenitrothion. First, further investigation into the fate of these pesticides as administered to chickens, in particular adult chickens, will provide a better overall picture of this species as a test subject. Secondly, due to the chemical degradation of the pesticides post-exposure, it would be appropriate to include their metabolites (particularly the fipronil sulfone) as test substances during *in ovo* testing. Lastly, it is important to look for potential

developmental effects in other oviparous species, especially given the high species variability in sensitivity to fipronil.

In order to assess the real risk of exposure of avian embryos to pesticides, a maternal transfer study needs to be carried out using adult breeding hens. The doses of the test substances in the current study were chosen across a broad, mostly logarithmic range in order to identify potential effects in a range likely to be experienced by embryos of females exposed to these pesticides. However, the dose an egg will actually receive depends on the amount transferred from its mother. This amount can be variable depending on the species and/or the test substance (Russell, Gobas and Haffner 1999). In addition, fipronil has been demonstrated to accumulate in variable amounts, even in species of the same taxa (Chaton et al. 2002). It is important to know the likelihood that the pesticide will be passed from the adult chicken to the egg, as well as the toxicological effects at various doses. This will provide a more complete picture of the effect of maternal exposure to these pesticides on offspring development.

Residue analysis of chicken egg yolk laid by dosed hens will provide a better idea of both the amount of pesticide transferred to the egg as well as its chemical nature. When a reproductive female is exposed to a contaminant, her body will transform some if not all of the pesticide before it is incorporated into yolk precursor lipids and passed on to the developing egg (Kubota et al. 2002). Because both fenitrothion and fipronil degrade rapidly *in vivo* (Hainzl and Casida 1996; Tsuda et al. 1997), the likelihood that the parent compound reaches the egg before biotransformation is reduced and the likelihood of deposition of metabolites is increased. The effects of the metabolites on

development may differ greatly from those of the parent compound and therefore independent investigation should be carried out as to their teratogenicity.

Further investigation is needed into the effects on selected species of native birds, especially those that differ in their modes of development, such as altricial or semi-altricial species. Eggs of the domestic chicken were used in the present study because galliform birds have been demonstrated to be highly sensitive to fipronil. Thus, the chicken embryo may be representative of galliforms found in the field (Tingle et al. 2003). Galliforms are also a precocial species and thus most of the developmental process occurs within the egg (Starck and Ricklefs 1998). In contrast, altricial birds hatch earlier in development and carry out the rest of their growth in the nest depending upon parental care for thermoregulation and nutrition.

While this study has focused primarily on one avian species, the toxicity to oviparous species from other taxa is also worth investigating. Locust control spray events occur at a time in Australia when many vertebrate species are breeding. Consequently, other oviparous species may also deposit fipronil or fenitrothion residues into their eggs along with the yolk lipid precursors. In particular, many reptilian species inhabit the areas over which fenitrothion and fipronil are sprayed (Cogger 2000; Bain et al. 2004). In regards to other contaminants, females within these taxa can bioaccumulate and transfer a higher concentration of certain lipophilic pollutants predicted from contact exposure, thus magnifying the dose delivered to their offspring and perhaps its toxic effect (Hopkins et al. 2004; Kadokami et al. 2004). It should be

expected that other lipid soluble molecules such as fipronil and fenitrothion could also be transferred to reptilian and anuran eggs. As these eggs are produced and often laid enmass, as opposed to serial production in birds, it is likely that the entire clutch could be affected.

Appendix I

Table A1.1 Comparative toxicity of fipronil across invertebrate species. h – hours post exposure; LD – lethal dose; LC – lethal concentration.

Table A1.2 Comparative toxicity of fipronil across vertebrate species. h – hours post exposure; LD – lethal dose; LC – lethal concentration.

Appendix II

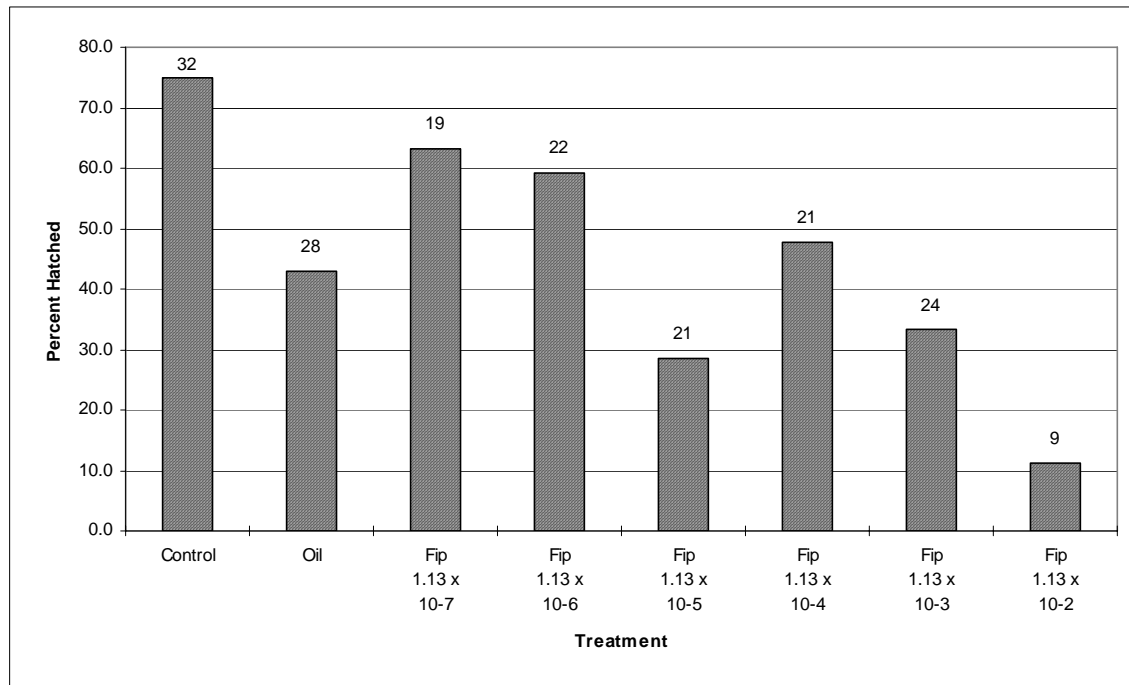


Figure A2.1 Percent hatchability (the number of eggs that hatched of all fertile eggs injected) unadjusted in domestic chicken eggs exposed to a single dose of fipronil *in ovo*. (Numbers above bars indicate sample size).

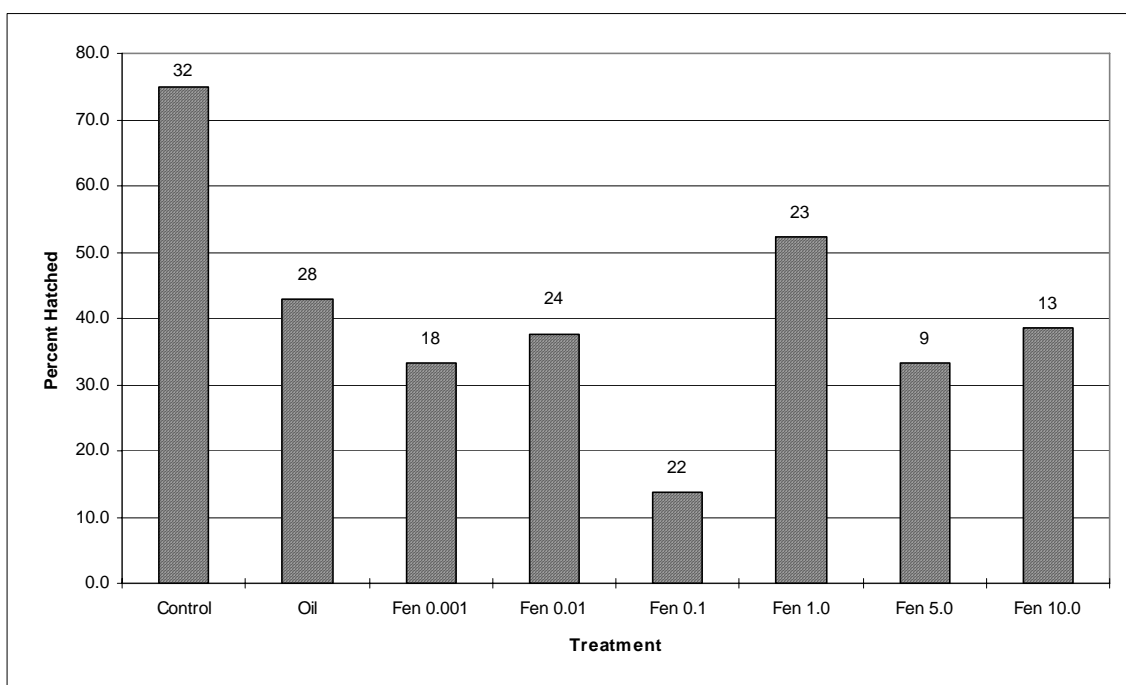


Figure A2.2 Percent hatchability (the number of eggs that hatched of all fertile eggs injected) unadjusted in domestic chicken eggs exposed to a single dose of fipronil *in ovo*. (Numbers above bars indicate sample size).

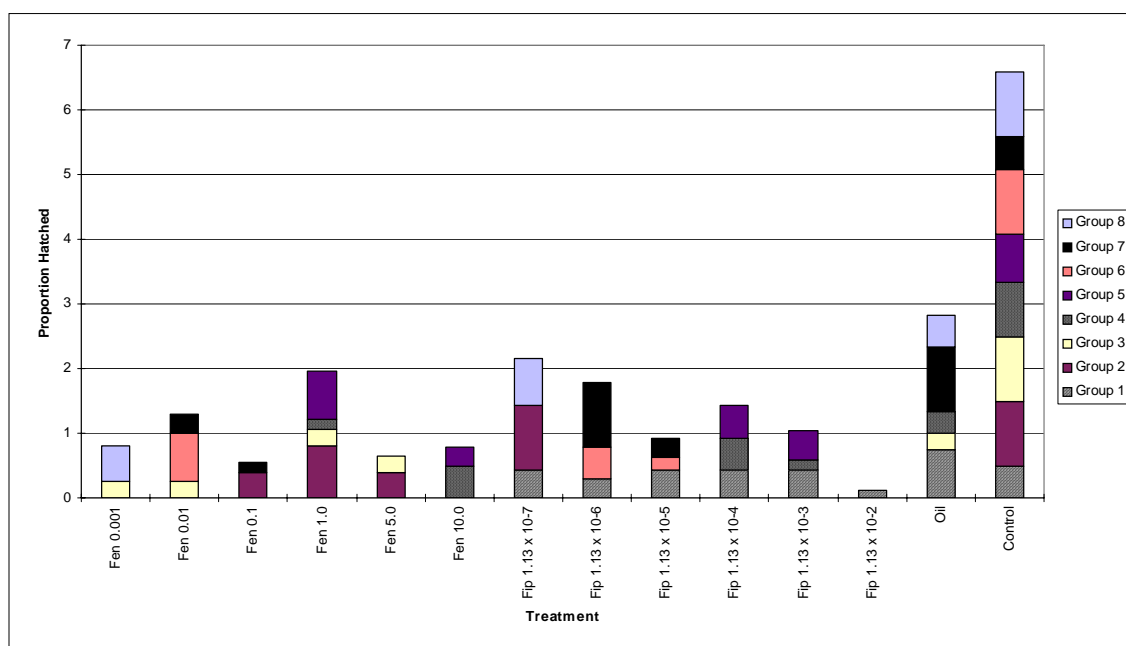


Figure A2.3 Hatchability rates divided by round of injection for eggs of the common chicken exposed to a single dose of either fipronil or fenitrothion *in ovo*.

Table A2.1: Descriptive statistics for excised femurs lengths taken 24 hours post-hatch in domestic chicken eggs exposed to a single dose of fenitrothion *in ovo*.

Treatment (mg/kg)	N	Mean	Std. Error
FEN 0.001	1	26.40	-
FEN 0.01	2	24.29	0.63
FEN 0.1	1	26.16	-
FEN 1.0	4	23.94	0.74
FEN 5.0	2	24.74	0.36
OIL	5	23.79	0.54
CONTROL	6	25.19	0.39

Appendix III

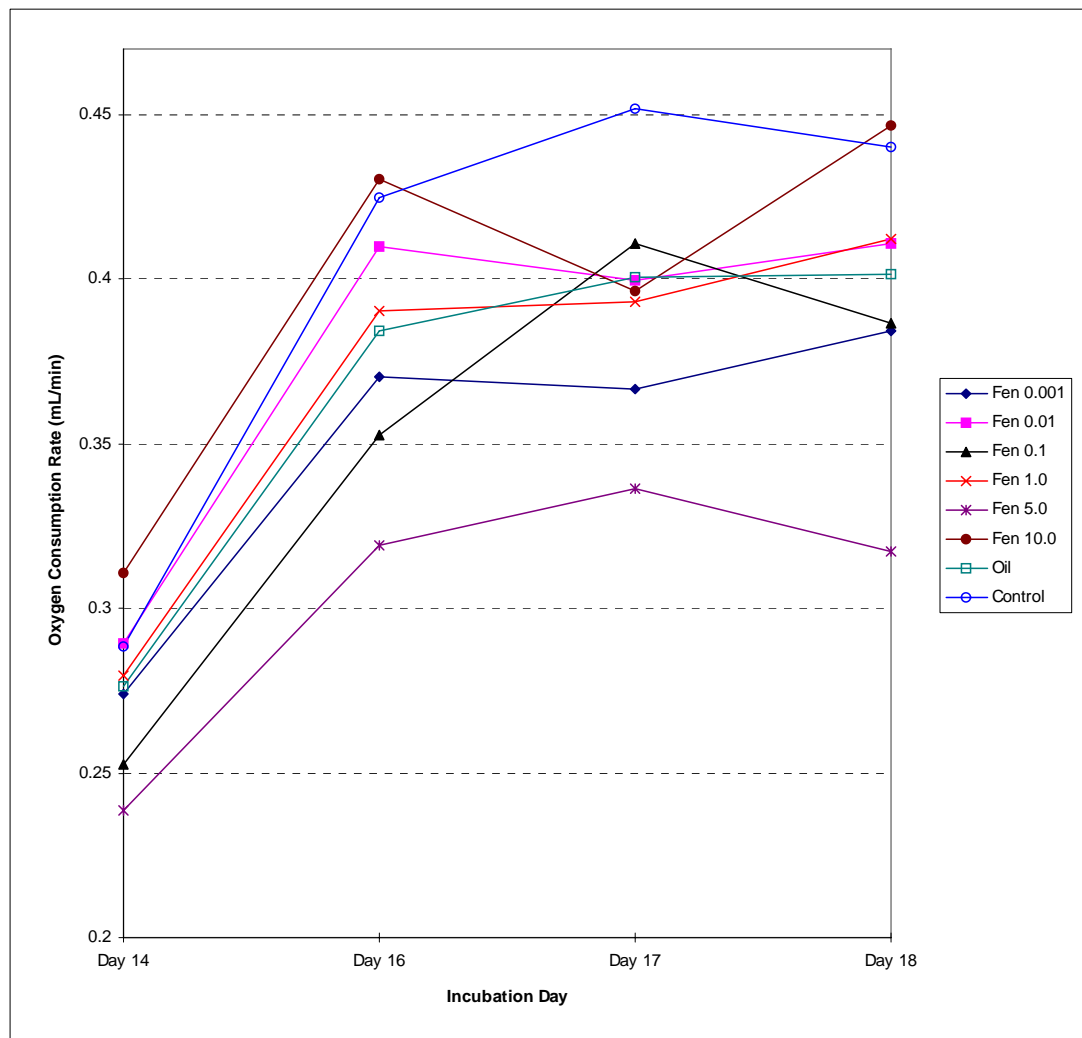


Figure A3.1 Mean oxygen consumption rate (mL/min) over time in domestic chicken eggs during incubation following an acute *in ovo* exposure to fenitrothion. Means are taken from all readings taken for embryos alive each day; see Table 1 for sample sizes and standard errors.

Table A3.1 Numbers and standard errors for mean oxygen consumption rate from all embryos alive each day during incubation in domestic chickens exposed to an acute *in ovo* dose of fenitrothion.

Treatment (mg/kg) / Incubation Day	Day 14	Day 16	Day 17	Day 18
Fen 0.001	N = 7; SE = 0.010	N = 7; SE = 0.011	N = 7; SE = 0.017	N = 7; SE = 0.018
Fen 0.01	N = 10; SE = 0.011	N = 8; SE = 0.014	N = 10; SE = 0.012	N = 10; SE = 0.013
Fen 0.1	N = 6; SE = 0.011	N = 5; SE = 0.032	N = 6; SE = 0.048	N = 6; SE = 0.035
Fen 1.0	N = 12; SE = 0.009	N = 12; SE = 0.020	N = 12; SE = 0.026	N = 12; SE = 0.019
Fen 5.0	N = 5; SE = 0.011	N = 5; SE = 0.020	N = 5; SE = 0.025	N = 5; SE = 0.037
Fen 10.0	N = 7; SE = 0.010	N = 5; SE = 0.025	N = 7; SE = 0.034	N = 7; SE = 0.018
Oil	N = 12; SE = 0.009	N = 10; SE = 0.010	N = 12; SE = 0.005	N = 12; SE = 0.011
Control	N = 21; SE = 0.008	N = 22; SE = 0.009	N = 24; SE = 0.009	N = 21; SE = 0.009

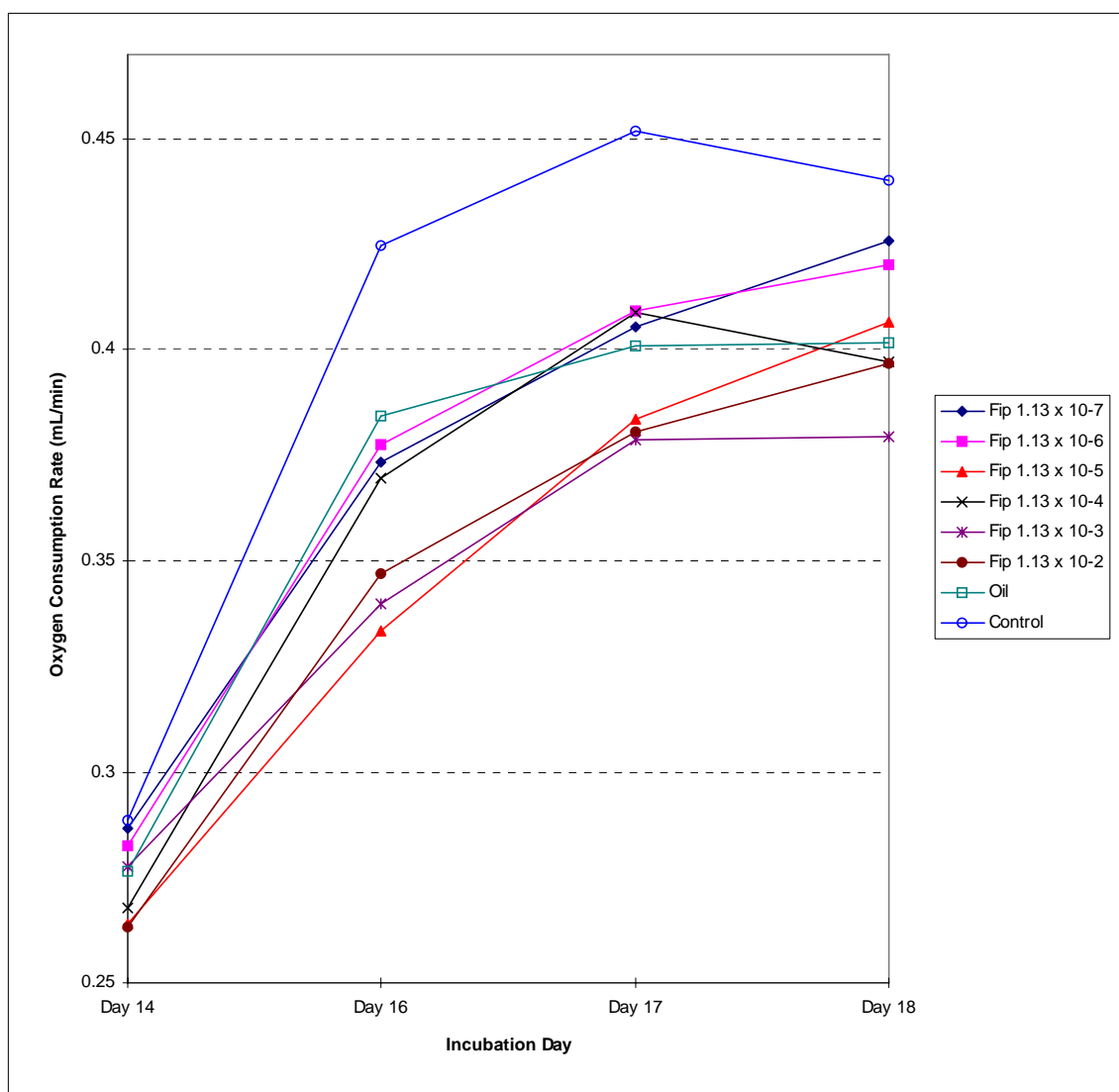


Figure A3.2 Mean oxygen consumption rate (mL/min) over time in domestic chicken eggs during incubation following an acute *in ovo* exposure to fipronil. Means are taken from all readings taken for embryos alive each day; see Table 2 for sample sizes and standard errors.

Table A3.2 Numbers and standard errors for mean oxygen consumption rate from all embryos alive each day during incubation in domestic chickens exposed to an acute *in ovo* dose of fipronil.

Treatment (mg/kg) / Incubation Day	Day 14	Day 16	Day 17	Day 18
Fip 1.13 X 10⁻²	N = 11; SE = 0.008	N = 11; SE = 0.011	N = 11; SE = 0.014	N = 10; SE = 0.007
Fip 1.13 X 10⁻³	N = 14; SE = 0.012	N = 7; SE = 0.029	N = 14; SE = 0.015	N = 14; SE = 0.016
Fip 1.13 X 10⁻⁴	N = 6; SE = 0.018	N = 4; SE = 0.028	N = 6; SE = 0.038	N = 6; SE = 0.030
Fip 1.13 X 10⁻⁵	N = 13; SE = 0.009	N = 13; SE = 0.013	N = 13; SE = 0.021	N = 13; SE = 0.019
Fip 1.13 X 10⁻⁶	N = 10; SE = 0.013	N = 10; SE = 0.022	N = 10; SE = 0.026	N = 10; SE = 0.030
Fip 1.13 X 10⁻⁷	N = 1	N = 1	N = 1	N = 1
Oil	N = 12; SE = 0.009	N = 10; SE = 0.010	N = 12; SE = 0.005	N = 12; SE = 0.011
Control	N = 21; SE = 0.008	N = 22; SE = 0.009	N = 24; SE = 0.009	N = 21; SE = 0.009

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