COMPARATIVE IN VIVO AND IN VITRO EFFECTS OF ORGANOPHOSPHORUS ANTICHOLINESTERASES IN THE OUTBRED CD-1 MOUSE AND GREAT PLAINS TOAD

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ANTICHOLINESTERASES IN THE OUTBRED CD-1

MOUSE AND GREAT PLAINS TOAD

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Abstract: Amphibians are generally less sensitive than mammals to the acute toxicity of organophosphorus (OP) insecticides, but the basis for these differences is unclear. This study compared *in vivo* sensitivity of a mammal (*Mus musculus*, CD-1 outbred) and an amphibian (the Great Plains toad, *Anaxyrus cognatus*) to chlorpyrifos oxon (CPO), the active metabolite of the organophosphorus pesticide chlorpyrifos. Following intraperitoneal dosing, adult male toads were about 13-fold less sensitive to CPO than adult male mice based on acute maximum tolerated doses (MTDs). At equi-toxic doses (0.6, 0.8 or 1 x MTD), brain acetylcholinesterase (AChE) inhibition was noted in mice but not in toads. In contrast, fatty acid amide hydrolase (FAAH) in liver was inhibited by CPO in both species. Carboxylesterase, a common enzyme involved with OP detoxification, was undetectable in toad brain. Toad brain AChE was about 15-fold less sensitive *in vitro* to inhibition by CPO than mouse enzyme (IC_{50}, 20 min at 37ºC: 136 vs 9 nM), roughly agreeing with *in vivo* differences in sensitivity. We used an indirect inhibition assay to evaluate possible species differences in target-site detoxification of CPO. Toad brain homogenate was markedly more effective at inactivating CPO *in vitro* than an equivalent amount of mouse brain. These data suggest that the relative insensitivity of toad brain AChE to CPO contributes to the resistance to acute toxicity and may be due to more effective target-site detoxification.

TABLE OF CONTENTS

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

Figure Page

CHAPTER I

INTRODUCTION

Organophosphorus (OP) compounds have been extensively used in agriculture, industry and public health over the last six decades (Grube et al., 2011; Atwood and Paisley-Jones, 2017). Some OPs have also been used as agents of warfare and terrorism. An OP insecticide (naled, 1,2-dibromo-2,2-dichloroethylphosphate) is currently being used to control *Aedes aegypti* in the US as the concern for an outbreak of the Zika virus increases, raising public concern over unwanted effects of insecticide use even in the face of a possible viral epidemic. The risks of untoward effects of OP pesticides to humans and the environment are legitimately weighed against the public health benefits of controlling insect vectors of disease (Pope et al., 2005). In contrast, the notorious use of OPs in chemical warfare and terrorism is of no benefit to society; concern over such uses continues today (Rosman et al., 2014).

OP compounds are most widely used as insecticides, contributing to a substantial portion (~33%) of insecticide use in the US (Atwood and Paisley-Jones, 2017). The OP insecticide chlorpyrifos (diethyl 3,5,6-trichloropyridinyl phosphorothioate), first introduced in 1965, is still widely used in the US and abroad (Giesy et al., 2014). According to USDA survey estimates, at least three million pounds of chlorpyrifos alone

were applied in the US in 2015 (USDA-NASS, 2016). Because OPs are used extensively as pesticides, they can be common environmental contaminants (Ecobichon, 2001; Gilliom, 2007; Giesy et al., 2014). While OP pesticides can be highly effective against "pests," their widespread use and presence in the environment creates opportunities for exposure and toxicity in non-target species.

The potential for OP exposure in wildlife necessitates the assessment of the risk OPs pose. One of the challenges faced in such risk assessments is interspecies variation in sensitivity. While direct comparisons of OP toxicity between species are relatively rare, the current literature demonstrates substantial variation in sensitivity to acute OP toxicity across numerous wildlife species, with the general trend that avian species are highly sensitive, mammals are moderately sensitive, and herpatofauna and piscine species are less sensitive (Wallace, 1992). Because the number of wildlife species is prohibitively great for comprehensive toxicity testing, risk assessment has traditionally relied on a small number of surrogate species. Therefore, understanding the factors that contribute to differential sensitivity may help us better understand phylogenetic differences between species as well as increase the validity of risk assessments.

The primary mechanism of acute OP toxicity is initiated by the inhibition of acetylcholinesterase (AChE), the enzyme responsible for the hydrolysis of the neurotransmitter acetylcholine (ACh) at cholinergic synapses (Quinn, 1987). Upon cholinergic neuron depolarization, ACh is released at the presynaptic terminal to activate cholinergic receptors on postsynaptic neurons, muscle cells or autonomic effector organs (Costa, 1988). Acetylcholinesterase, one of the most catalytically efficient enzymes in the

body, is strategically located within cholinergic synapses to degrade ACh and terminate cholinergic signaling.

Organophosphorus anticholinesterases interact with AChE (and other serine hydrolases) in a similar manner as the natural substrate (Aldridge and Reiner, 1972). The active site serine hydroxyl group engages in nucleophilic attack on the carbon atom of ACh or the phosphorus atom of an OP, leading to acyl intermediates in both cases (Richardson et al., 2009). With the substrate ACh, hydrolysis of the acyl intermediate (and consequent reactivation of the enzyme) occurs very rapidly. When OPs are bound, however, hydrolysis of the acyl intermediate is exceedingly slow, leading to long-term inactivation of the enzyme (Aldridge and Reiner, 1972). It should be noted that given sufficient time, the phosphylated enzyme will reactivate, i.e., it is generally not "irreversibly" inhibited, even though OPs are routinely referred to as irreversible AChE inhibitors. Spontaneous reactivation of the inhibited enzyme is frequently so slow that recovery of ACh hydrolysis following OP intoxication is primarily mediated by *de novo* synthesis of new enzyme molecules (Main, 1964). In some cases, however, the bound OP-enzyme complex can undergo an "aging" reaction that strengthens the phosphorus-enzyme bond and prevents reactivation, leaving the enzyme now indeed irreversibly inhibited (Richardson et al., 2009).

Cholinergic signaling participates in a wide range of functions throughout the nervous system of vertebrates and invertebrates (Massoulie and Bon, 1982). When an OP is covalently bound to the active site serine residue of AChE, the enzyme can no longer catalyze the hydrolysis of ACh. Sufficient inhibition of enzymes within the synapses leads to ACh accumulation and excessive stimulation of cholinergic receptors (Fukuto,

1990). Classic signs of acute cholinergic toxicity with extensive AChE inhibition can be separated into peripheral and central nervous system effects. Excessive stimulation of muscarinic receptors within the peripheral nervous system may increase autonomic secretions (salivation, lacrimation, urination and defecation; SLUD signs) and induce bradycardia and miosis (Gaines, 1960; Pope, 2006). Tremors and muscle fasciculations are caused by over-stimulation of nicotinic receptors in the peripheral nervous system. Additionally, cholinergic disruption in the central nervous system can result in seizure, coma, neuropathology and respiratory depression (Pope, 2006; Richardson et al., 2009; Pouliot et al., 2016). In mammals, lethality from severe OP poisoning is generally the result of asphyxiation caused by disruption of respiratory control in centers within the pons-medulla, excessive airway secretions and paralysis of the respiratory muscles (De Candole et al., 1953; Albuquerque et al., 1985).

The majority of OP pesticides are applied as "parent" compounds, requiring metabolic activation by mixed function oxidases (e.g., cytochrome P450s, flavin-containing monooxygenases) for potent biocidal activity (Potter and O'Brien, 1964; Fukuto, 1990). For example, the insecticide chlorpyrifos has little anticholinesterase activity until converted by oxidative desulfuration to a much more reactive oxygen analog (i.e., chlorpyrifos oxon [3,5,6-trichloro-2-pyridinyl phosphate], Levi and Hodgson, 1992). Following metabolic activation of the parent OP compound the oxon is much more potent at inactivating AChE and other sensitive enzymes. Differential metabolic activation across species is one of many factors that contribute to differential sensitivity (Potter and O'Brien, 1964; Hitchcock and Murphy, 1971; Chambers and Carr, 1995; Boone and Chambers, 1997). Species that more effectively activate parent OP

compounds generally have higher levels of toxic active metabolites, which can mediate higher sensitivity to cholinergic toxicity.

The variation in metabolic activation of parent compounds among species is somewhat consistent with, but cannot completely explain, differences in sensitivity. Despite variation within taxonomic groupings (Hitchcock and Murphy, 1971; Lavado and Schlenk, 2011), metabolic activation of OPs is generally more rapid in liver tissue of birds and mammals than in fish (Potter and O'Brien, 1964; Hitchcock and Murphy, 1971; Machin et al., 1975; Wallace and Dargan, 1987; Chambers and Carr, 1995). Differences in metabolic activation of OPs have also been reported between age groups within a single species. For example, juvenile rats have lesser metabolic activation towards OPs compared to adults; however, they also have lower detoxification capacity that contributes to their ultimately higher sensitivity (Pope et al., 1991; Zhang et al., 2002). The same trend of maturational decreases in sensitivity has also been reported in avian species. Wolfe and Kendall (1998) reported that nestlings of both European starlings (*Sturnus vulgaris*) and red-winged blackbirds (*Agelaius phoeniceus*) are more sensitive to OP intoxication compared to adults; however, the underlying mechanism of this difference is still unclear.

The chemical structure of an OP can also influence metabolism. Certain OPs can be preferentially metabolized by cytochrome P450s or monooxygenases, partially explaining why some OP insecticides are more toxic than others (Sultatos et al., 1985; Wallace and Kemp, 1991). For example, mallard (*Anas platyrhynchos*) liver enzymes preferentially bioactivate methyl parathion relative to parathion and fenitrothion (Thompson et al., 1995). Variation in chemical structure also influences OP activation in

mammals (Machin et al., 1975; Chambers and Carr, 1995) and fish (Lavado and Schlenk, 2011). For example, mosquitofish (*Gambusia affinis*) liver microsomes bioactivate chlorpyrifos more readily than parathion (Boone and Chambers, 1997).

Metabolic activation of OPs alone cannot explain the differences in sensitivity among species. If sensitivity was strictly related to metabolic activation, one would expect rodents to be the most sensitive group, as they typically have higher rates of activation for OPs *in vitro* (Hitchcock and Murphy, 1971; Poet et al., 2003). This is not the case, however, as avian species with comparatively lower bio-activation rates are still more sensitive compared to rodents *in vivo* (Wallace, 1992; Barron and Woodburn, 1995; Crane et al., 2016). Together, this evidence suggests that other factors can have a prominent role in determining differential sensitivity to OP toxicity.

Detoxification processes can determine how quickly OPs are inactivated, and thus influence species sensitivity to OP toxicity. Oxons are hydrolyzed primarily by non-specific esterases. Aldridge (1953) defined esterases able to hydrolyze OPs as "A-esterases," e.g., paraoxonase. Esterases that are inhibited by OPs were termed "B-esterases," e.g., acetylcholinesterase and butyrylcholinesterase. The relative activities of A-esterases in mammalian tissues have been exhaustively investigated as they are implicated in the detoxification of nerve agents and pesticides in humans (Li et al., 2005). Mammals generally express higher A-esterase levels than other taxonomic groups (Chambers and Carr, 1995). In contrast, birds are generally low in plasma A-esterase levels compared to mammals, which correlates with their higher sensitivity to OPs (Brealy et al., 1980; Wolfe and Kendall, 1998; Crane et al., 2016). Conversely, the role of A-esterases in sensitivity of amphibians and reptiles has not been extensively

investigated. Some studies suggested that amphibians have higher levels of A-esterases, allowing them to effectively inactivate OPs (Edery and Schartzberg-Porath, 1960). Despite relatively high serum A-esterase levels, mammals tend to be more sensitive to OP intoxication than fish and herpatofauna (Murphy, 1966; Barron and Woodburn, 1995). This suggests that, while detoxification is clearly an important factor in determining sensitivity, A-esterases are not the primary determinant of species sensitivity to OP intoxication.

The relative density of non-target B-esterases (i.e., OP-sensitive enzymes other than AChE) can also play a role in comparative sensitivity. Acetylcholinesterase is a member of the serine hydrolase superfamily of enzymes, which share a common "catalytic triad" of residues at the active site (for review, see Bachovochin and Cravatt, 2012). Butyrylcholinesterase (BChE; the "sister" enzyme to AChE) and carboxylesterases (CbE) are other serine hydrolases capable of binding to and inactivating OP insecticides (Lockridge, 2015). Several additional serine hydrolases, such as the two primary endocannabinoid-degrading enzymes, monoacylglycerol lipase (MAGL) and fatty acid amide hydrolase (FAAH) are also sensitive to covalent modification by OPs and may contribute to non-AChE consequences of OP intoxication (Nallapaneni et al., 2008; Pope et al., 2010). While none of these enzymes are capable of hydrolyzing OPs, they present stoichiometric binding sites that can sequester toxic OP molecules and lessen AChE inhibition. Genomic studies reveal that the endocannabinoid system is highly conserved in vertebrates (for review, see Cottone et al., 2008; Fezza, 2014), but its function has not been extensively investigated in non-mammalian models, including amphibians. As such, these enzymes and their possible contribution to

differential sensitivity have not been investigated in amphibians exposed to OP insecticides.

While differences in metabolic activation and detoxification of OPs can contribute to interspecies variation in sensitivity, it is accepted that interactions at the target site (i.e., toxicodynamic factors) are the most important determinant of species sensitivity (Wallace, 1992). The complete reaction from free to phosphylated enzyme is described by the bimolecular rate constant (k_i); once phosphylated, the enzyme-OP complex can either age (becoming irreversibly inhibited) or undergo spontaneous reactivation (Richardson et al., 2009). Comparative kinetic studies suggest that there are substantial interspecies differences in the kinetics of AChE inhibition by OPs, with the more sensitive species displaying more rapid phosphylation (Andersen et al., 1977; Wang and Murphy, 1982; Worek et al., 2011; Herket et al., 2011). Differences in chemical structures of OP compounds can also influence inhibition kinetics. For example, the rapid aging of the soman-AChE complex increases the nerve agent's lethality compared to other anticholinesterases because of its resistance to reactivation (Berry and Davies, 1966). The k_i of AChE inhibition in the nervous system can predict relative sensitivity to OP toxicity.

Several studies have reported that amphibians can be markedly less sensitive to acute OP toxicity compared to other taxonomic groups (Hawkins and Mendel, 1946; Edery and Schatzberg-Porath, 1960; Andersen et al., 1972; Hall and Kolbe, 1980; Shapira et al., 1998; Crane et al., 2016). The molecular basis for such differences in sensitivity among classes of vertebrates remains unclear. Amphibians are important members of many ecosystems, generally representing a large portion of the vertebrate biomass (Kerby

et al., 2010). Amphibians can often be found in and around agricultural landscapes, in part due to the widespread application and accumulation of water for irrigation, as well as to the transformation of habitats into farmland (Marsh et al., 2004; Knutson et al., 2004; Mazerolle et al., 2005). While many studies have attempted to link pesticide use with amphibian population declines (Sparling et al., 2001; Sparling and Fellers, 2009), terrestrial amphibians are often similarly or less sensitive than other wildlife species to some common organic insecticides (Kerby et al., 2010). In the case of OP insecticides, amphibians are generally markedly less sensitive than birds and mammals (Wang and Murphy, 1982; Crane et al., 2016). Most toxicological research investigating the effects of OPs in amphibians has been done in tadpoles and as such was focused more on developmental toxicity (for review, see Pechen de D'Angelo and Venturino 2005). This focus may be justifiable given that immature amphibians (tadpoles and metamorphs) are considered the most vulnerable subpopulation, but that emphasis has limited the understanding of differences in sensitivity in adults and their potential role in population changes.

Edery and Schatzberh-Porath (1960) reported that toad (*Bufo viridis*) and frog (*Pelophylax ridibundus*) had up to 360-fold higher LD_{50} values compared to mouse (*Mus musculus*) after subcutaneous exposure to the OPs paraoxon or diisopropylfluorophosphate (DFP). Hudson and coworkers (1984) noted a much lower difference in sensitivity between amphibians and mammals with the OP pesticide chlorpyrifos, i.e., the oral LD_{50} for chlorpyrifos in American bullfrogs (*Rana catesbeiana*) was only 2.6-fold higher than in rats (*Rattus norvegicus)*. Barron and Woodburn (1995) reported that the acute oral LD_{50} for chlorpyrifos was about 10-fold

higher in American bullfrogs than mallard ducks (*Anas platyrhynchos*). A recent meta-analysis of acute oral toxicity by Crane and coworkers (2016) concluded that adult amphibians are generally less sensitive than birds and mammals to acute OP toxicity.

Only a few studies have evaluated potential mechanisms that might contribute to differences in sensitivity between amphibians and mammals. Hawkins and Mendel (1946) reported that brain AChE from grass frog (*Rana pipiens*) was about 200-fold less sensitive to inhibition *in vitro* by the carbamate anticholinesterase physostigmine, compared to inhibition of the enzyme in brain tissue from either horse (*Equus ferus*) or mouse (*Mus musculus*). Edery and Schatzberh-Porath (1960) proposed that more effective detoxification by CbE may be important in the lower sensitivity exhibited by amphibians. More recently, Robles-Mendoza and coworkers (2011) noted that CbE in juvenile axolotls (*Ambystoma mexicanum*) was less sensitive than AChE to inhibition *in vivo* by chlorpyrifos. Attademo and coworkers (2017) suggested that intestinal CbE might contribute to detoxification of chlorpyrifos in tadpoles (R*hinella arenarum*). The relative prevalence of non-target A- and B- esterases in amphibians and their contribution to sensitivity has not been resolved.

Other investigators studied differences in sensitivity of AChE to inhibition by OP anticholinesterases in differential toxicity. Zahavi and coworkers (1971) investigated the basis for differential sensitivity to OP toxicity in toad (*Bufo viridis*), medfly (*Ceratitis capitata*), American cockroach (*Periplaneta americana*) and several strains of mites (*Tetranychus cinnabarinus*). They found that enzymes from species that had relatively low sensitivity *in vivo* (toads and OP-resistant mites) exhibited different substrate kinetics (i.e., lower V_{max} and higher K_m), which led to the proposal that the relative sensitivity of

AChE to anticholinesterses was correlated with structural differences in the active site region of the enzyme, which may in turn influence inhibitor interactions with the catalytic site.

Shapira and coworkers (1998) studied acetylcholinesterase inhibition and acute toxicity in wild type *Xenopus laevis* larvae and mutants expressing human AChE. Interestingly, larvae expressing the human enzyme were more sensitive to acute paraoxon toxicity than the wild type larvae. *In vitro* differences in sensitivity to inhibition between the frog wild type and human enzyme were also noted with other inhibitors including pyridostigmine, DFP and tacrine. Shapira and coworkers (1998) also compared substrate inhibition, i.e., the reduction of enzyme activity in the presence of high concentrations of endogenous substrate (Reed et al., 2010), between wildtype *X. laevis* and human enzymes. Kinetic analysis suggested a lack of substrate inhibition with the wildtype enzyme but not with the human enzyme. Thus, work by Zahavi and coworkers (1971), Shapira and colleagues (1998) suggest structural differences in AChE may lead to differences in substrate kinetics, which may in turn influence the relative resistance of amphibian AChE to inhibition by OPs.

In summary, there are a variety of factors that can contribute to species differences in sensitivity to OPs. Comparative toxicity studies can improve risk assessment of pesticides and further our understanding of physiological differences between species. Despite their ecological significance and recent population declines, relatively little research has been conducted on acute OP toxicity in adult amphibians. A number of reports indicate that adult amphibians are relatively insensitive to acute OP toxicity compared to mammals, and that several factors could play a role. This project

compared acute sensitivity of a mammal (*Mus musculus*) and an amphibian (*Anaxyrus cognatus*) to chlorpyrifos oxon and investigated toxicodynamic and toxicokinetic factors that may contribute to amphibians' reportedly low sensitivity. The specific aims were:

1. to compare *in vivo* toxicity in mouse and toad following acute chlorpyrifos oxon exposure.
2. to compare *in vitro* interactions of OP-sensitive enzymes with substrate and selected inhibitors.
3. to screen for possible OP-binding proteins in mouse and toad brain that may contribute to differential toxicity.

CHAPTER II

METHODS

Chemicals

Chlorpyrifos oxon (O, O'-diethyl O-3, 5, 6-trichloro-2-pyridinyl phosphate; CPO), paraoxon (O, O'-diethyl O-4 nitrophenyl phosphate) and naled (dimethyl-1, 2-dibromo-2,2-dichlorethyl phosphate) were purchased from ChemService (West Chester, PA). Diisopropylfluorophosphate (DFP) was kindly provided by Derik Heiss at Batelle Memorial Institute (Columbus, OH). Stock solutions (10 mM) of these inhibitors were prepared in ethanol and stored desiccated under nitrogen at -70°C. 2-Arachidonoyl thioglycerol was purchased from Cayman Chemical Company (Ann Arbor, MI). FP-Rhodamine (TAMRA-FP serine hydrolase probe) was purchased from Thermo Fisher Scientific (Waltham, MA). Coomassie Brilliant blue (R 250), glycine and kaleidoscope protein ladder were purchased from Bio-Rad (Hercules, CA). Octanoyl-methoxypyridine was a generous gift from Dr. Bruce Hammock at University of California (Davis, CA). Peanut oil was from Lou Ana Oils (Brea, CA). Acetylthiocholine and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Animals

Male outbred CD-1 mice (*Mus musculus*; eight weeks of age) were purchased from Charles River (Wilmington, MA). Mice were maintained in shoebox cages with standard rodent chow and water available *ad libitum* under a 12-12 hour light-dark cycle. Sexually mature male and female *Anaxyrus cognatus* were hand-collected from Blaine, Logan, Garfield and Kingfisher Counties, OK during April and May 2015-6. Toads were transported back to an animal housing facility at Oklahoma State University and kept in same-sex groups in 75 L aquaria with moist sand ($\geq$ 5 cm deep). Toads were fed weekly with mealworms (*Tenebrio molitor*) *ad libitum* dusted with Miner-All (Sticky Tongues Farm, Sun City, CA) and provided continuous access to bowls of dechlorinated water under 12-12 hour light-dark cycle. All animals were acclimated to housing conditions for at least five days prior to dosing or tissue collection for *in vitro* studies. Toads were euthanized by immersion in buffered MS-222 (tricaine methane sulfonate, 0.5%, pH 7) followed by decapitation. Mice were euthanized by decapitation using a guillotine. Brain and liver tissues were collected from both species and frozen at -70°C for subsequent biochemical assays. All procedures were approved by the Oklahoma State University Institutional Animal Care and Use Committee and conducted in AAALAC accredited facilities.

Maximum Tolerated Dose Estimation

To compare mouse and toad sensitivity to CPO, we estimated maximum tolerated doses (MTD). Rate of OP activation (i.e., conversion of parent insecticide to active metabolite) can influence relative sensitivity between groups (Chambers and Carr 1995). As there is little information on the relative activation of OPs between mammals and

amphibians, we used CPO (the active oxon metabolite of chlorpyrifos) for comparative toxicity studies. An up-and-down method was used to estimate CPO toxicity (Bruce et al., 1987). One animal was treated (intraperitoneal injection; ip) with an initial dose (10 mg/kg for mice; 100 mg/kg for toads; dissolved in peanut oil, 3 mL/kg). If the animal survived for 24 hours, the subsequent animal received 1.3 x that dose. If the first dose was lethal, the next animal was given 0.77 x the first dose. This process was repeated until an animal died (going up in dose) or survived (going down in dose), yielding an MTD. This estimate was used in the following acute toxicity studies. Morbidity in toads was evaluated through loss of righting reflex and gross evaluation of cardiac chest movements.

Acute Toxicity

Toads and mice were divided into four experimental groups: 0, 0.6, 0.8 and 1 x the respective MTD. The control groups received only vehicle (peanut oil; 3 mL/kg). Animals were weighed, dosed and monitored at regular intervals (0.5, 1, 2, 4, 8 and 24 hours). Four hours after dosing, animals were scored for involuntary movements following the methods of Moser and coworkers (1995), modified by Liu and Pope (1996). For involuntary movements, animals were scored as follows: 2, normal movements; 3, mild fine tremors or head and forelimbs; 4, whole-body tremors; 5, myoclonic jerks. For SLUD signs, animals were scored as follows: 1, normal (no secretions); 2, one or two mild secretions; 3, multiple moderate secretions; 4, multiple severe secretions. Twenty-four hours after dosing, animals were weighed and sacrificed.

In acute lethality studies, we observed that the toads became hypo-active and unresponsive to handling. To quantify possible loss of coordination in a uniform manner that could be compared between species, we designed a "tilt test." A shoebox cage (28 x 17 x 12 cm) was attached to a platform on a hinge, allowing the cage to be tilted in a continuous manner. Four hours after dosing, animals were individually placed in the cage that was then raised steadily along the hinge until the animal either: 1) lost balance and fell to the lower end of cage or 2) moved to the lower end of the cage on their own volition. For consistency, animals were always placed facing away from the hinge and towards the "upper" (the end that would be raised) end of the cage. The maximum positive angle (°) each animal maintained was measured using a protractor. Trials were video recorded and scored by an investigator blinded to the treatment groups. We expected that intoxicated animals would be less coordinated and therefore slide to the bottom of the cage at lower inclines than control animals.

Biochemical Assays

Brain and liver tissues were homogenized in ice-cold phosphate-buffered saline, pH 7 at 30,000 RPM for 20 seconds using a Polytron PT 3100 (Kinematica AG; Switzerland) prior to enzyme assays. Liver homogenates were centrifuged at 1,000 x g at 4°C for 10 min using an Avanti J-25I centrifuge (Beckman Coulter; Indianapolis, IN), and supernatants were used for subsequent assays.

Acetylcholinesterase (brain), BChE (brain and liver) and MAGL (brain and liver) activities were measured using a photometric method similar to Ellman et al. (1961) and Ulloa and Deutsch (2010) as modified by Liu and Pope (2015). A cocktail of substrate

(final concentrations: acetylthiolcholine, 1 mM; butyrylthiocholine, 1 mM; 2-arachidonoyl thioglycerol, 0.1 mM) and DTNB (0.1 mM final) was added to tissue homogenates with 10 mM Tris buffer with 1 mM EDTA (pH 7.2; Tris-E) in a final reaction volume of 200 µl and activity was measured kinetically at 412 nm for 5 min at 37°C using a SpectraMax 340 PC plate reader (Molecular Devices; Sunnyvale, CA). Carboxylesterase (brain and liver) activity was measured following the method of Clement and Erhardt (1990) as modified by Chanda and coworkers (1997). Substrate (p-nitrophenyl acetate; 250 µM final) was added to tissue homogenates with Tris-E buffer (pH 7.2) in a final reaction volume of 200 µl and activity was monitored kinetically at 405 nm for 5 min at 37°C.

Fatty acid amide hydrolase (brain and liver) activity was measured following the fluorometric method of Huang et al. (2007). Tissues were incubated with the substrate octanoyl-methoxypyridine (50 µM final) in 125 mM sodium phosphate buffer (pH 8, 1% glycerol, 0.1% Triton X-100) in a final reaction volume of 200 µl for 30 min at 37°C. Formation of hydrolysis product was measured fluorometrically (excitation: 320 nm, emission: 396 nm) using a SpectraMax M2 plate reader (Molecular Devices; Sunnyvale, CA). All enzyme activities were normalized by protein content using the method described by Bradford (1976).

In vitro sensitivity of mouse and toad AChE to OP inhibitors was determined by first pre-incubating tissue homogenates with vehicle or one of a range of OP concentrations (CPO: 0.5 – 1,000 nM; paraoxon: 1 – 50,000 nM; naled: 1 – 5,000 nM) at 37°C for 20 min, and then measurement of residual activity as described above. Control samples were pre-incubated with vehicle (ethanol; 0.5% final, v/v) to estimate 100%

activity. IC_{50} (the concentration of inhibitor that depresses enzyme activity by 50%) values were determined through non-linear regression (log inhibitor vs. % control-variable slope: four parameters) using GraphPad Prism (Version 6.0). Some assays compared sensitivity of mouse and toad AChE to CPO (0.5 – 1,000 nM) at 26°C (20 min) in order to evaluate the influence of temperature on interactions between enzymes and inhibitors.

Substrate Kinetics

Substrate kinetic parameters (k_m and V_{max}) for acetylcholinesterase were compared by incubating tissues (37°C) with varying concentrations of acetylthiocholine (10 – 1,000 µM) and monitoring results kinetically (10 min, 37°C). Parameters were determined through non-linear regression of enzyme velocity vs substrate concentration, according to the Michaelis-Menten equation using GraphPad Prism (Version 6.0).

OP Inactivation Assay

The ability of a tissue to detoxify OP compounds was measured by an indirect inhibition assay (Chanda et al., 1997; Karanth et al., 2001). Either buffer or tissue homogenate (2.5 mg tissue) was pre-incubated (15 min; 37°C) with varying concentrations of CPO (3 – 800 nM final) in 200 mM Tris-HCl buffer (pH 7.8) in a final volume of 250 µl. A marker enzyme (purified recombinant human BChE; 250 ng) was then added and further incubated for 20 min (37°C). Residual BChE activity was then measured photometrically as described above. Residual BChE activity was used as an indicator of tissue OP inactivation.

Screening for OP-Sensitive Proteins in Brain Tissue

To identify potential enzymes that may contribute to differential OP inactivation in mouse and toad brain tissue, we used a fluorescent serine hydrolase probe as described by Nomura and Casida (2011). In brief, 2 mL of 1:25 brain tissue homogenate was centrifuged at 1,000 x g for 10 min (4°C) using an Avanti J-25I centrifuge (Beckman Coulter; Indianapolis, IN). The supernatant was removed and spun again at 10,000 x g for 10 min (4°C). The resulting membrane pellet (P_2) was homogenized in 200 µl ice-cold 50 mM Tris-HCl (pH 7.4) and pre-incubated with vehicle (ethanol, 0.5% final, v/v) or CPO (1, 10, 100 nM final; 20 min; 37°C). FP-rhodamine (4 µM final) was then added and incubated for 30 min (23°C). The reaction was stopped by the addition of loading buffer (30 µl) and then heat inactivated at 90°C for 10 min. Ten percent polyacrylamide gels (0.1% SDS) with 4.5% stacking gels, 1 mm thick, were poured in a Mini-protean Tetra cell casting stand (Bio-Rad; Hercules, CA). Samples (10 µl/well) were loaded and separated at 120 V for two hours using a Mini-protean Tetra PowerPac Universal supply system (Bio-Rad; Hercules, CA).

Gels were fluorometrically visualized using a Cy 3 filter (excitation: 552 nm, emission: 575 nm) with an Amersham Imager 600 (GE Healthcare Bio-Sciences; Pittsburg, PA). FP-rhodamine labels serine hydrolases, so the absence of fluorescent bands in CPO-exposed tissues can indicate CPO-sensitive proteins. Fluorescent probe gels were stained by washing in 50% methanol, 10% acetic acid, 25% Coomassie Brilliant blue (R 250; w/v) with shaking for one hour and then de-stained in 10% acetic with shaking for eight hours. CPO-sensitive bands from toad brain were excised, digested and proteins were identified using LC/MS-MS.

Data Analysis

All *in vivo* enzyme and tilt test data were presented as mean ± SEM and were analyzed for significance by one-way analysis of variance (ANOVA); significant main effects were followed by *post hoc* Dunnett's multiple comparisons. Involuntary movement and SLUD scores were presented as median with interquartile range and were analyzed for significance using a Kruskal-Wallace one-way ANOVA. IC_{50} values for single inhibitors and inactivation assays were presented as mean and 95% confidence intervals. Substrate kinetic constants (k_m, V_{max}) were presented as mean ± SEM and compared between species using an unpaired Student's T-test. In all comparisons, $p < 0.05$ was used to indicate statistical significance. All statistical approaches were conducted using GraphPad Prism (software version 6.0).

CHAPTER III

RESULTS

Maximum Tolerated Dose

Toads were markedly less sensitive to CPO than mice. The maximum tolerated dose of CPO was 13-fold higher in toads than in mice (77 vs 5.9 mg/kg). Functional signs of toxicity were noted in both species treated with respective MTDs (summarized in Table 1). In mice, we observed pronounced involuntary movements, muscular fasciculations and SLUD signs at doses greater than 4.7 mg/kg. During acute lethality studies, toads (n = 2) that received 100 mg/kg of chlorpyrifos oxon displayed pronounced miosis and parotoid secretions (both thick white and thin clear secretions); however, these signs were not observed with lower doses. In toads, no involuntary movements, tremors or muscle fasciculations were noted. Instead, we observed hypo-activity and excessive salivation at the highest dose (100 mg/kg).

Acute Toxicity

The acute toxicity study used increments of the respective MTD (0, 0.6, 0.8 and 1 x MTD). Mice received 0, 3.6, 4.7 or 5.9 mg/kg CPO, while toads received 0, 46, 62 or 77 mg/kg CPO. In mice, there was significant, dose-related inhibition of brain AChE ($F_{3,4}$ = 15.1 , $p < 0.0001$; Figure 1), BChE ($F_{3,4} = 9.27$, $p = 0.0009$; Figure 2), MAGL ($F_{3,4}$ = 3.58, $p = 0.03$; Figure 3), FAAH ($F_{3,4}$ =19.4, $p < 0.0001$; Figure 4) and CbE ($F_{3,4} = 10.6$, $p = 0.0004$; Figure 5). In contrast, there was no significant change in any enzyme in toad brain. Even in the highest dose group (77 mg/kg), we observed no significant effect on toad brain AChE ($F_{3,3} = 0.369$, $p = 0.78$; Figure 1), BChE ($F_{3,3} = 0.105$, $p = 0.96$; Figure 2) or MAGL ($F_{3,3} = 0.227$, $p = 0.88$; Figure 3). There was a significant main effect of treatment on toad brain FAAH ($F_{3,3} = 5.68$, $p = 0.012$; Figure 4), but no significant pairwise comparisons were noted. Carboxylesterase activity was undetectable in all control and treated toad brains.

In liver, CPO did not inhibit BChE activity in either mouse ($F_{3,4} = 0.437$, $p = 0.734$) or toad ($F_{3,3} = 1.45$, $p = 0.28$; Figure 6). Fatty acid amide hydrolase activity was significantly inhibited in the livers of both mice ($F_{3,4} = 12.8$, $p = 0.0002$) and toads ($F_{3,3} = 6.92$, $p = 0.0059$; Figure 7). Neither mouse ($F_{3,4} = 0.691$, $p = 0.57$) nor toad liver CbE ($F_{3,3} = 2.58$, $p = 0.10$; Figure 8) was inhibited by CPO. Of note, basal CbE activity in mouse liver was >100-fold higher compared to activity in toad liver.

Four hours after dosing, we evaluated the animals for involuntary movements and SLUD signs and conducted the tilt test. In mice, 4.7 and 5.9 mg/kg CPO elicited involuntary movements ($p = 0.004$), while no involuntary movements were noted in any

toads (Figure 9). The higher dose (5.9 mg/kg CPO) induced SLUD signs in mice ($p = 0.02$), while no SLUD signs were noted in toads (Figure 10). Mice and toads also responded differently in the tilt test. In toads, CPO exposure was associated with a higher incline with 62 and 77 mg/kg CPO ($p = 0.02$; Figure 11). With the controls and animals treated with the lowest dose (46 mg/kg), toads rapidly hopped to the lower end of the box, while at the two higher doses, toads failed to react and instead splayed their limbs and slid down the bottom surface of the box when the incline was sufficiently steep. Chlorpyrifos oxon did not influence the incline at which mice slid to the cage bottom ($F_{3,4} = 2.26$, $p = 0.12$; Figure 11).

Sign	Mouse (10 mg/kg CPO)	Toad (100 mg/kg CPO)
Tremors	+	-
Salivation	+	+
Lacrimation	+	-
Urination	+	-
Defecation	+	-
Miosis	-	+
Erythema	-	+
Parotoid Secretions	NA	+

Table 1. Toxic signs in mouse and toad following single lethal dose of chlorpyrifos oxon (CPO). There were substantial differences in toxic signs between mice and toads. These data represent observations made during acute lethality studies. + indicates that the functional response was observed within 24 hours of dosing, while – indicates no response (n = 2).

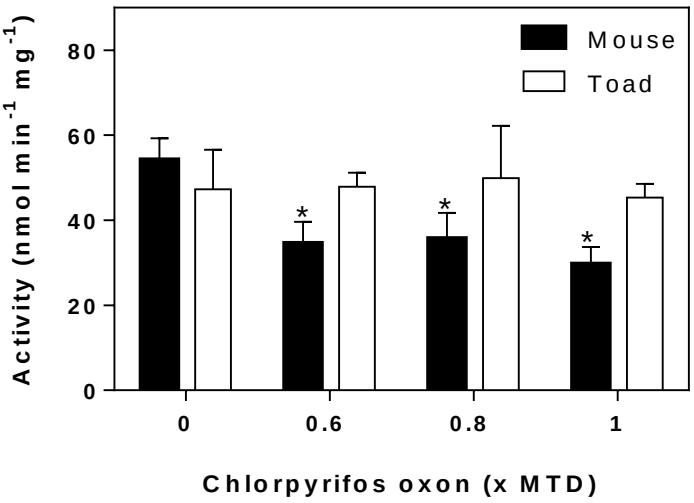

Figure 1. Mouse and toad brain acetylcholinesterase (AChE) activities following acute chlorpyrifos oxon (CPO) exposure (ip). Mice were treated with either vehicle (peanut oil), 3.6, 4.7 or 5.9 mg/kg CPO. Toads were treated with either vehicle (peanut oil), 46, 62 or 77 mg/kg CPO. CPO significantly inhibited brain AChE in mice, but had no significant effect on toad brain AChE. Data represent mean specific activity $\pm$ SEM (mouse: n = 5; toad: n = 4). Asterisks indicate a significant difference compared to vehicle controls ($p < 0.05$).

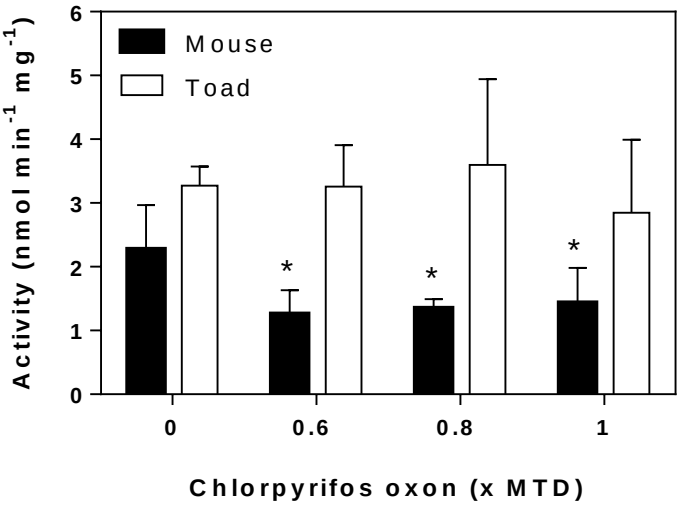

Figure 2. Mouse and toad brain butyrylcholinesterase (BChE) activities following acute chlorpyrifos oxon (CPO) exposure (ip). Mice were treated with either vehicle (peanut oil), 3.6, 4.7 or 5.9 mg/kg CPO. Toads were treated with either vehicle (peanut oil), 46, 62 or 77 mg/kg CPO. CPO significantly inhibited brain BChE in mice, but had no significant effect on toad brain BChE. Data represent mean specific activity $\pm$ SEM (mouse: n = 5; toad: n = 4). Asterisks indicate a significant difference compared to vehicle controls ($p < 0.05$).

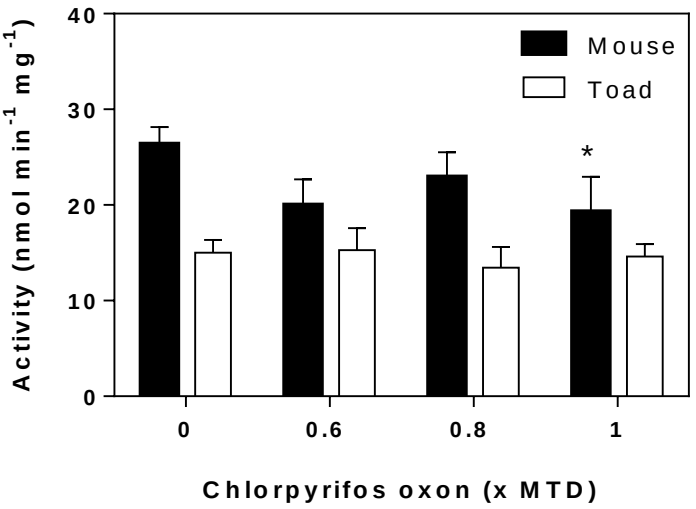

Figure 3. Mouse and toad brain monoacylglycerol lipase (MAGL) activities following acute chlorpyrifos oxon (CPO) exposure (ip). Mice were treated with either vehicle (peanut oil), 3.6, 4.7 or 5.9 mg/kg CPO. Toads were treated with either vehicle (peanut oil), 46, 62 or 77 mg/kg CPO. Chlorpyrifos oxon significantly inhibited brain MAGL in mice that received 5.9 mg/kg (1 x MTD), but had no significant effect on toad brain MAGL. Data represent mean specific activity $\pm$ SEM (mouse: n = 5; toad: n = 4). Asterisk indicates a significant difference compared to vehicle control ($p < 0.05$).

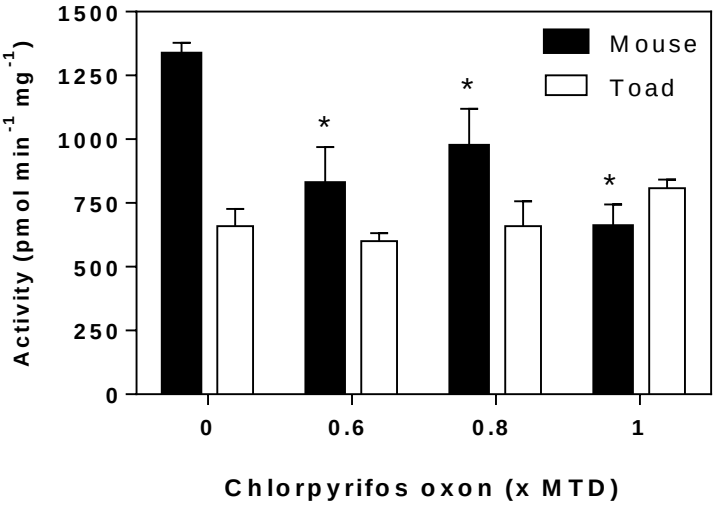

Figure 4. Mouse and toad brain fatty acid amide hydrolase (FAAH) activities following acute chlorpyrifos oxon (CPO) exposure (ip). Mice were treated with either vehicle (peanut oil), 3.6, 4.7 or 5.9 mg/kg CPO. Toads were treated with either vehicle (peanut oil), 46, 62 or 77 mg/kg CPO. CPO significantly inhibited brain FAAH in mice, but had no significant effect on toad brain FAAH. Data represent mean specific activity $\pm$ SEM (mouse: n = 5; toad: n = 4). Asterisks indicate a significant difference compared to vehicle controls ($p < 0.05$).

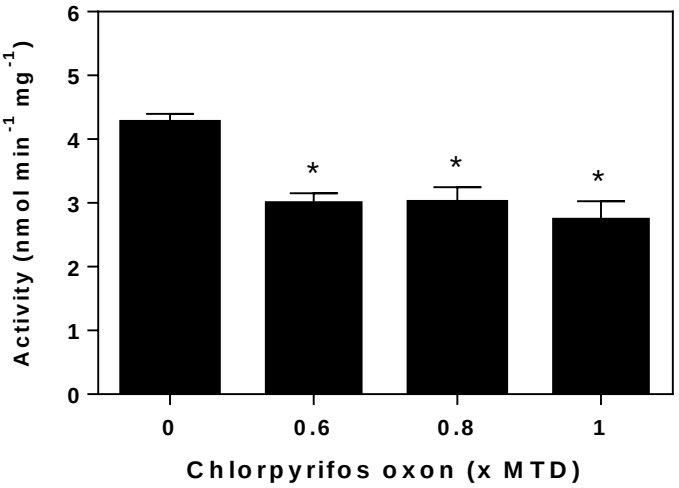

Figure 5. Mouse brain carboxylesterase (CbE) activity following acute chlorpyrifos oxon (CPO) exposure (ip). Mice were treated with either vehicle (peanut oil), 3.6, 4.7 or 5.9 mg/kg CPO. Toads were treated with either vehicle (peanut oil), 46, 62 or 77 mg/kg CPO. CPO significantly inhibited brain CbE in mice. Carboxylesterase activity was undetectable in all toad brains. Data represent mean specific activity $\pm$ SEM (n = 5). Asterisks indicate a significant difference compared to vehicle control ($p < 0.05$).

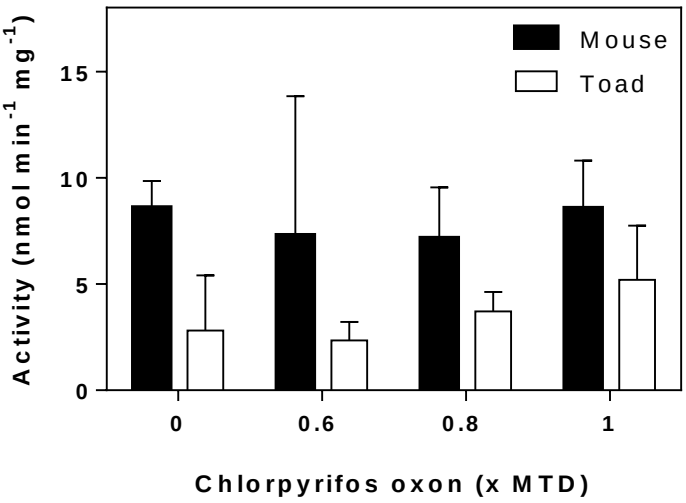

Figure 6. Mouse and toad liver butyrylcholinesterase (BChE) activities following acute chlorpyrifos oxon (CPO) exposure (ip). Mice were treated with either vehicle (peanut oil), 3.6, 4.7 or 5.9 mg/kg CPO. Toads were treated with either vehicle (peanut oil), 46, 62 or 77 mg/kg CPO. CPO had no significant effect on liver BChE of either species. Data represent mean specific activity $\pm$ SEM (mouse: n = 5; toad: n = 4).

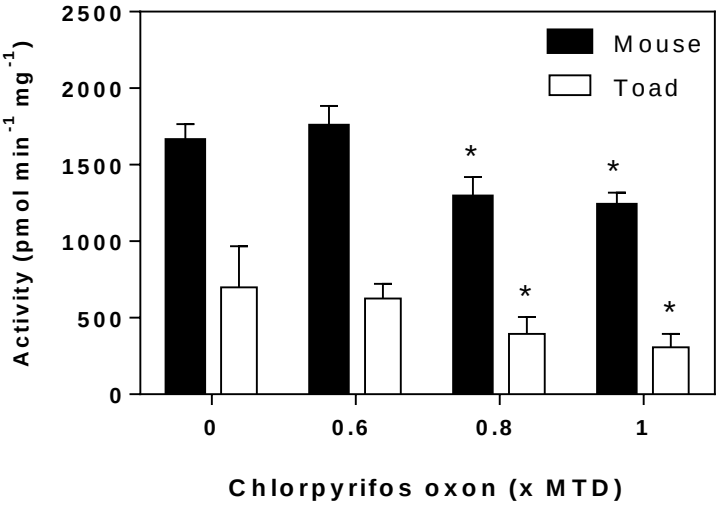

Figure 7. Mouse and toad liver fatty acid amide hydrolase (FAAH) activities following acute chlorpyrifos oxon (CPO) exposure (ip). Mice were treated with either vehicle (peanut oil), 3.6, 4.7 or 5.9 mg/kg CPO. Toads were treated with either vehicle (peanut oil), 46, 62 or 77 mg/kg CPO. CPO significantly inhibited FAAH activities in the highest dose groups in both species. Data represent mean specific activity $\pm$ SEM (mouse: n = 5; toad: n = 4). Asterisks indicate a significant difference compared to vehicle controls ($p < 0.05$).

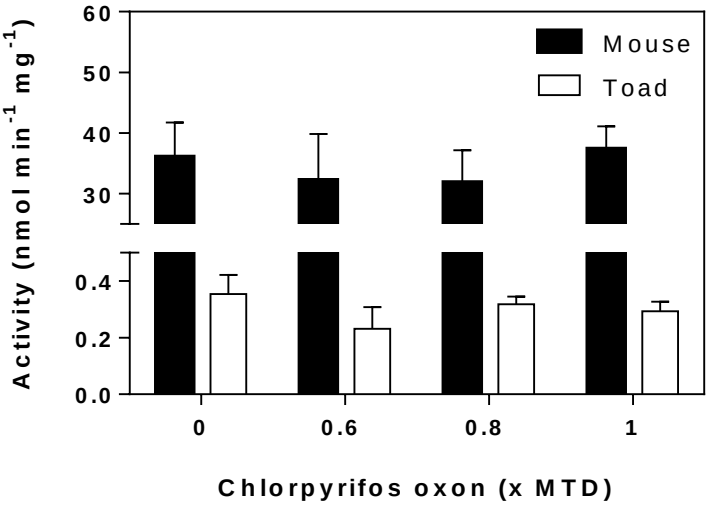

Figure 8. Mouse and toad liver carboxylesterase (CbE) activities following acute chlorpyrifos oxon (CPO) exposure (ip). Mice were treated with either vehicle (peanut oil), 3.6, 4.7 or 5.9 mg/kg CPO. Toads were treated with either vehicle (peanut oil), 46, 62 or 77 mg/kg CPO. CPO had no significant effect on liver CbE of either species. Data represent mean specific activity $\pm$ SEM (mouse: n = 5; toad: n = 4).

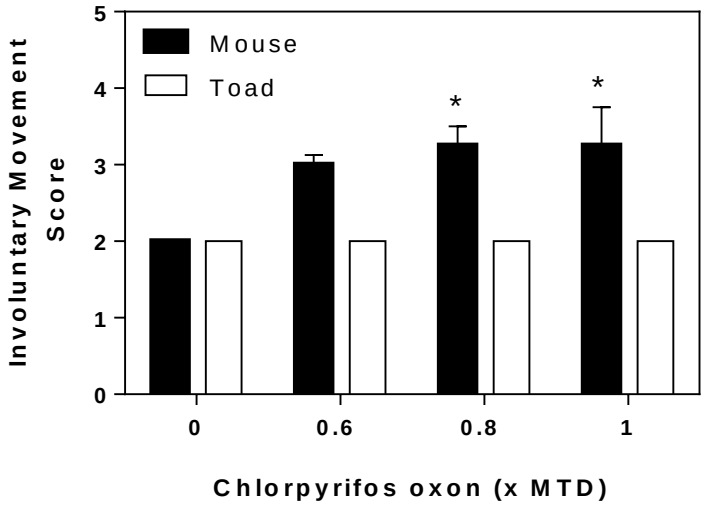

Figure 9. Involuntary movements in mice and toads following acute chlorpyrifos oxon (CPO) exposure (ip). Mice were treated with either vehicle (peanut oil), 3.6, 4.7 or 5.9 mg/kg CPO. Toads were treated with either vehicle (peanut oil), 46, 62 or 77 mg/kg CPO. CPO significantly increased involuntary movements in mice, but did not cause any involuntary movements in toads. Data represent median score with interquartile range (mouse: n = 5, toad: n = 4). Asterisks indicate significant difference compared to vehicle controls ($p < 0.05$).

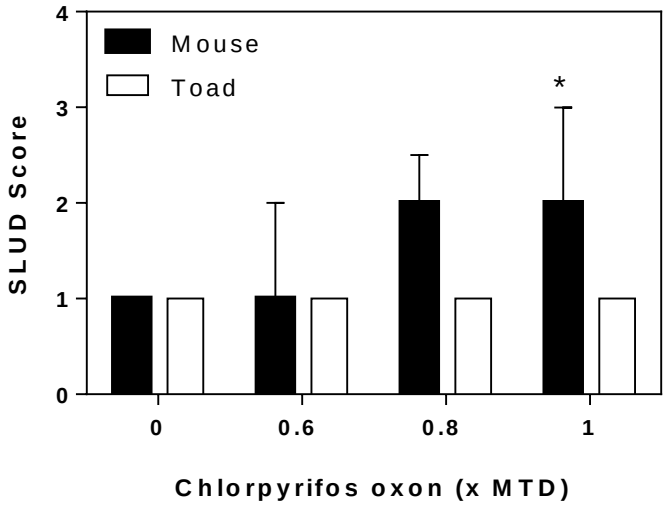

Figure 10. Autonomic secretions in mice and toads following acute chlorpyrifos oxon (CPO) exposure (ip). Mice were treated with either vehicle (peanut oil), 3.6, 4.7 or 5.9 mg/kg CPO. Toads were treated with either vehicle (peanut oil), 46, 62 or 77 mg/kg CPO. CPO significantly increased SLUD (acronym for salivation, lacrimation, urination and defecation) signs in mice, but did not cause any SLUD signs in toads. Data represent median score with interquartile range (mouse: n = 5, toad: n = 4). Asterisks indicate significant difference compared to vehicle controls ($p < 0.05$).

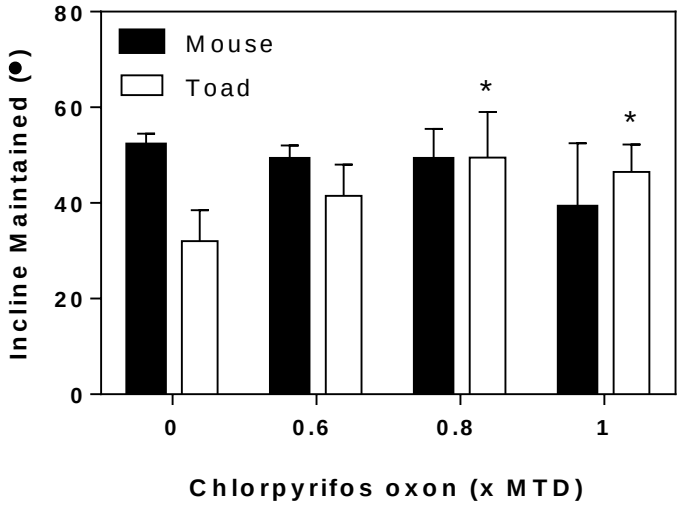

Figure 11. Stability of mice and toads in tilt test following acute chlorpyrifos oxon (CPO) exposure (ip). Mice were treated with either vehicle (peanut oil), 3.6, 4.7 or 5.9 mg/kg CPO. Toads were treated with either vehicle (peanut oil), 46, 62 or 77 mg/kg CPO. CPO significantly increased stability in toads, but had no effect on stability in mice. Data represent mean incline maintained $\pm$ SEM (mouse: n = 5; toad: n = 4). Asterisks indicate significant difference compared to vehicle controls ($p < 0.05$).

In vitro Studies

The IC_{50} values are summarized in Table 2. In all cases, AChE in toad tissues was markedly less sensitive to inhibition *in vitro* compared to mouse tissues. IC_{50} values in toad brain were 15-fold higher with CPO (Figure 12), 367-fold higher with paraoxon (Figure 13) and 32-fold higher with naled (Figure 14) compared to those in mouse brain tissue. Control AChE activities at 26°C were reduced compared to 37°C in both species. The IC_{50} for toad brain AChE with CPO at 26°C was 37-fold greater compared to mouse (Figure 15). In toad, the IC_{50} with CPO at 26°C was significantly higher compared to the IC_{50} at 37°C (226 vs 136 nM; $p = 0.001$). In contrast, there was no difference between the mouse brain AChE IC_{50} with CPO at 26 and 37°C (6.7 vs 9.0 nM; $p = 0.26$).

While there was no significant difference in brain AChE V_{max} between mouse (1.1 $\pm$ 0.055 nmol min^{-1}) and toad (0.90 $\pm$ 0.075; $p = 0.49$), K_m for toad (0.23 $\pm$ 0.054 mM) was 5-fold higher compared to mouse (0.043 $\pm$ 0.0094 mM; $p = 0.002$; Figure 16).

Species	Chlorpyrifos oxon - 37°C (nM)	Chlorpyrifos oxon - 26°C (nM)	Paraoxon - 37°C (nM)	Naled - 37°C (nM)
Mouse	9.00 (6.98 – 11.6)	6.70 (6.08 – 7.40)	15.7 (11.8 – 21.0)	53.8 (50.0 – 58.3)
Toad	136 (108 – 172)	226 (159 – 320)	5756 (5088 – 6511)	1705 (1401 – 2075)

Table 2. Sensitivity of acetylcholinesterase (AChE) in mouse and toad brain to inhibition by selected inhibitors *in vitro*. IC_{50} values are shown as mean (95% confidence interval). Toad brain AChE exhibits higher IC_{50} values for all inhibitors. Data are from 3 independent replicates (n = 4 for paraoxon in toad brain).

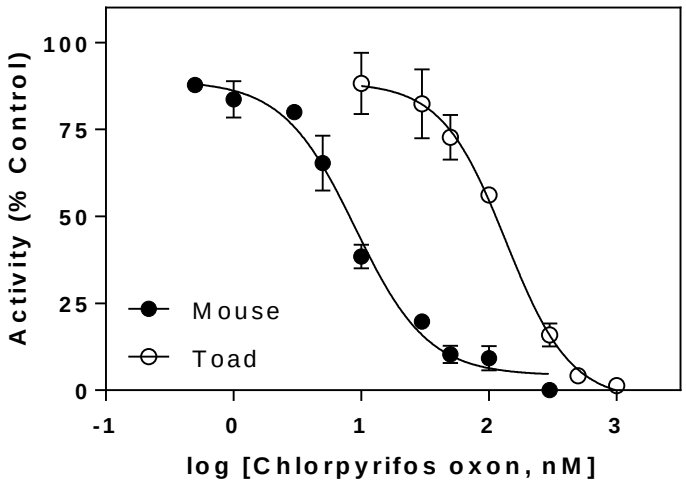

Figure 12. Comparison of mouse and toad brain acetylcholinesterase (AChE) inhibition by chlorpyrifos oxon (CPO) *in vitro*. Tissues were pre-incubated (37°C, 20 min) with either vehicle or varying concentrations of CPO (0.3 – 500 nM) and residual activity measured as described in methods. Data are expressed as % of control values (mouse: 0.32 $\pm$ 0.024 nmol/min; toad: 0.32 $\pm$ 0.026 nmol/min). Data represent mean $\pm$ SEM (n = 3 for each species). Mean IC_{50} (95% confidence interval) for mouse: 9.00 (6.98 – 11.6) nM; toad: 136 (108 – 172) nM.

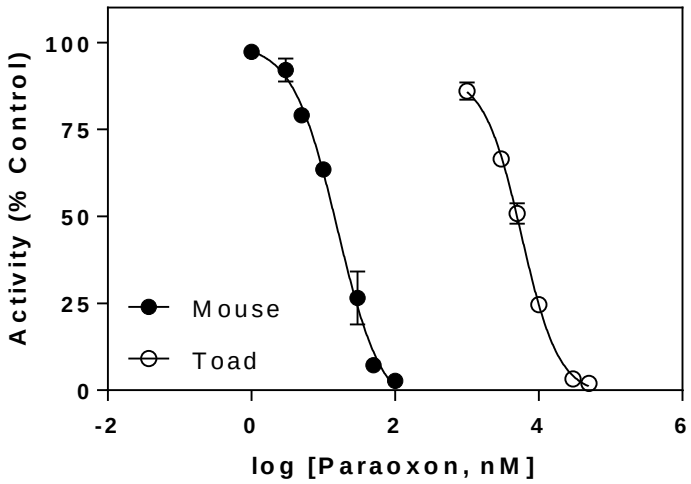

Figure 13. Comparison of mouse and toad brain acetylcholinesterase (AChE) inhibition by paraoxon (PO) *in vitro*. Tissues were pre-incubated (37°C, 20 min) with either vehicle or varying concentrations of PO (1 – 50,000 nM) and residual activity measured as described in methods. Data are expressed as % of control values (mouse: 0.35 $\pm$ 0.026 nmol/min; toad: 0.36 $\pm$ 0.050 nmol/min). Data represent the mean $\pm$ SEM (mouse: n = 3; toad: n = 4). Mean IC_{50} (95% confidence interval) for mouse: 15.7 (11.8 – 21.0) nM; toad: 5756 (5088 – 6511) nM.

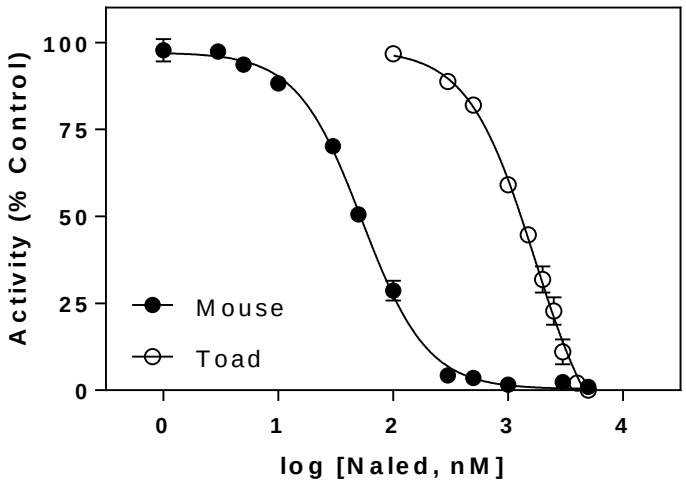

Figure 14. Comparison of mouse and toad brain acetylcholinesterase (AChE) inhibition by naled *in vitro*. Tissues were pre-incubated (37°C, 20 min) with either vehicle or varying concentrations of naled (1 – 5000 nM) and residual activity measured as described in methods. Data are expressed as % of control values (mouse: 0.36 $\pm$ 0.10 nmol/min; toad: 0.22 $\pm$ 0.0049 nmol/min). Data represent mean $\pm$ SEM (n = 3 for each species). Mean IC_{50} (95% confidence interval) for mouse: 53.8 (49.6 – 58.3) nM; toad: 1705 (1401 – 2075) nM.

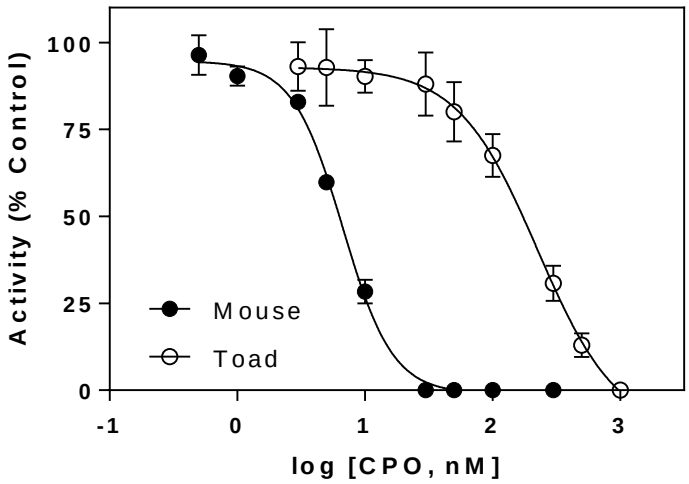

Figure 15. Comparison of mouse and toad brain acetylcholinesterase (AChE) inhibition by chlorpyrifos oxon (CPO) *in vitro* at 26°C. Tissues were pre-incubated (26°C, 20 min) with either vehicle or varying concentrations of CPO (0.3 – 500 nM) and residual activity measured as described in methods. Data are expressed as % of control values (mouse: 0.1997 $\pm$ 0.00465 nmol/min; toad: 0.1939 $\pm$ 0.01399 nmol/min). Data represent mean $\pm$ SEM (n = 3 for each species). Mean IC_{50} (95% confidence interval) for mouse AChE: 6.70 (6.08 – 7.40) nM; toad AChE: 226 (159 – 320) nM.

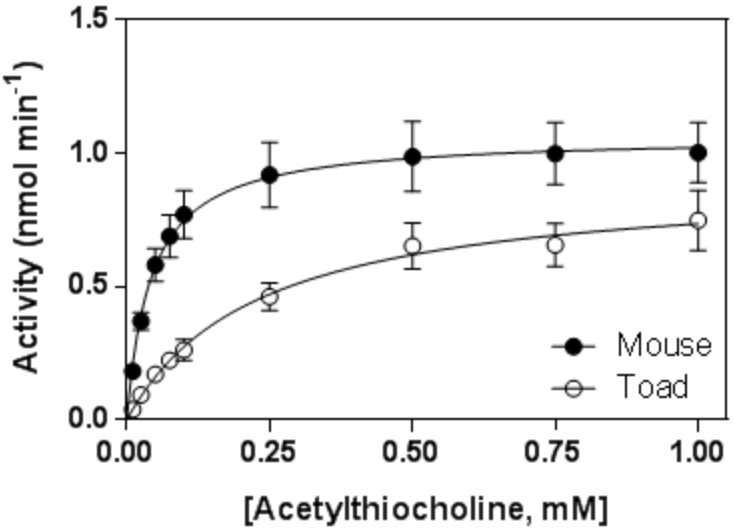

Figure 16. Kinetic evaluation of substrate hydrolysis by mouse and toad brain acetylcholinesterase (AChE) *in vitro*. Tissues were incubated with varying concentrations of acetylthiocholine (0.01 – 1 mM) and AChE activity was measured as described in methods. Data represent the mean $\pm$ SEM (mouse: n = 4, toad: n = 3). There was no significant difference in V_{max} between mouse (1.1 $\pm$ 0.055 nmol min^{-1}) and toad (0.90 $\pm$ 0.075; p = 0.49). K_m for toad (0.23 $\pm$ 0.054 mM) was significantly higher compared to mouse (0.043 $\pm$ 0.0094 mM; p = 0.002).

OP Inactivation Assay and OP-Sensitive Proteins

Mouse and toad brain homogenates were pre-incubated with varying concentrations of CPO. The ability of each tissue to inactivate CPO was then evaluated by adding purified BChE as a marker enzyme and measuring residual activity. Toad brain homogenate was substantially more effective at detoxifying CPO than mouse brain (Figure 17). Pre-incubation with toad brain tissue increased the IC_{50} for BChE with CPO by roughly 118-fold: from 31.5 (28.2 – 35.1) nM to 3,698 (very wide) nM, while pre-incubation with mouse brain only increased IC_{50} for BChE to 45.2 (40.1 – 51.0) nM.

We screened brain tissues of mouse and toad for CPO-sensitive proteins using a fluorescent serine hydrolase probe. Toad brain contains at least one protein (~50 kD) that is sensitive to binding CPO at 10 nM. This protein was not observed in mouse brain (Figure 18).

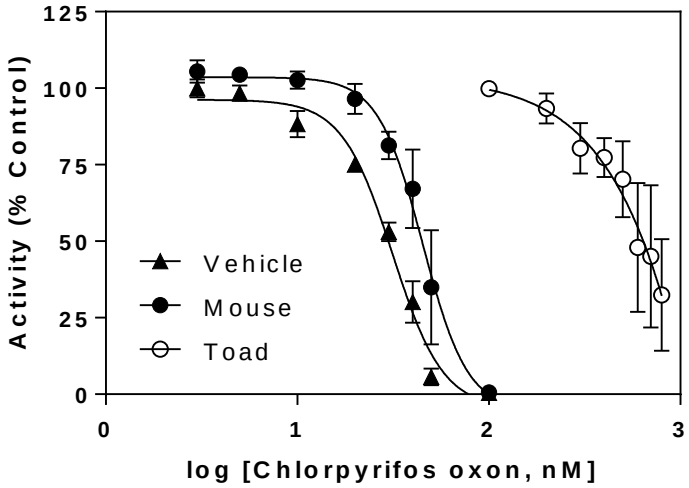

Figure 17. Inactivation of chlorpyrifos oxon (CPO) by mouse and toad brain homogenate. Mouse and toad brain tissues were incubated with either buffer or one of a range of CPO concentrations (3 – 800 nM). A marker enzyme (purified recombinant human BChE, 250 ng) was then added and residual activity was measured as described in the methods. Toad brain tissue was more effective at inactivating CPO than tissue from mouse. Data are presented as mean $\pm$ SEM (n = 3 for each group).

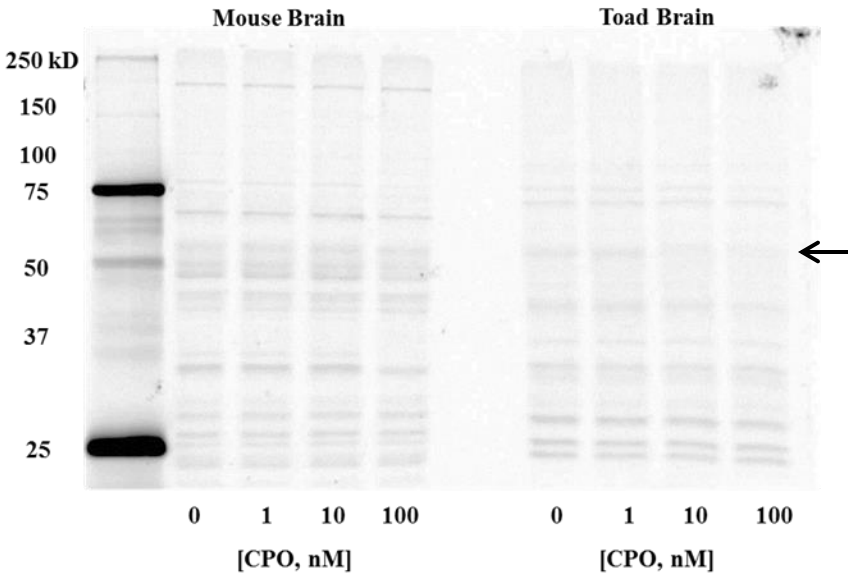

Figure 18. *In vitro* effects of chlorpyrifos oxon (CPO) on organophosphate-sensitive proteins in mouse and toad brain. Mouse and toad brain membrane pellets were incubated with either vehicle or varying concentrations of CPO (1, 10, 100 nM). A fluorescent serine hydrolase marker (FP-rhodamine, 4 µM final) was then added and incubated for 30 min (23°C). Samples (10 µl/well) were separated on a 10% polyacrylamide gel (0.1% SDS) with a 4.5% stacking gel at 120 V for two hours as described in methods. Gels were fluorometrically visualized using a Cy 3 filter (excitation: 552 nm, emission: 575 nm). Fluorescent bands suggest the presence of a serine hydrolase; decrease in band density suggests binding by CPO. Toad brain contains a CPO-sensitive protein (~50 kD, marked by arrow) not observed in mouse brain.

CHAPTER IV

DISCUSSION

In vivo Comparisons

Toads tolerated much higher doses of CPO than mice (MTDs: 77 vs. 5.9 mg/kg). At equi-toxic doses (based on MTD), mice displayed dose-related brain AChE inhibition, while there was no inhibition of toad brain AChE. The lack of AChE inhibition in treated toads suggests that toads are less sensitive than mice to acute OP toxicity because AChE is not inhibited by CPO. While we also observed dose-related inhibition of BChE, MAGL, FAAH and CbE in mouse brain, there was no inhibition of any of these enzymes in toad brain, suggesting that toads more readily detoxify CPO compared to mice. Previous studies have suggested that CbE plays a role in the inactivation of OPs in amphibians (Caballero de Castro et al., 1991; Attademo et al., 2014; Attademo et al., 2017), but the lack of CbE in toad brain and the relatively low liver activity compared to mice in our study suggests that CbE does not play a prominent role in inactivation of CPO in toads. Furthermore, toad liver CbE was not affected by CPO, consistent with the findings of Robles-Mendoza et al., (2011), who reported that CbE in juvenile axolotls was less sensitive than AChE to chlorpyrifos *in vivo*.

Interestingly, liver FAAH activity was significantly reduced by CPO in both species. Fatty acid amide hydrolase inhibition in toad liver might suggest that FAAH is more sensitive than AChE in toads. Because toad brain FAAH was unaffected by CPO, it is unlikely that FAAH is responsible for "protecting" brain AChE by binding to CPO. Previous studies indicated that in rats, DFP was a more potent *in vivo* FAAH inhibitor than expected based on *in vitro* sensitivity, implying an indirect modulation of FAAH may occur (Nallapaneni et al., 2006; Nallapaneni et al., 2008). It is possible that CPO toxicity indirectly depresses toad liver FAAH activity rather than via phosphylation and direct inhibition. Future studies should investigate FAAH as a sensitive endpoint for OP toxicity in amphibians.

There were also differences in functional signs between treated mice and toads. At equi-toxic doses (based on MTDs), mice displayed classic signs of cholinergic toxicity while few signs were noted in toads. In mice that received high doses of CPO, we noted increased tremors/involuntary movements consistent with previous reports of acute OP toxicity (Gaines, 1960; Liu and Pope, 1996). Though some authors have reported tremors in bullfrogs (*Rana catesbeiana*) following DFP exposure (Edery and Schatzberg-Porath, 1960), we did not observe any involuntary movements or muscle fasciculations in any toads. Instead, toads treated with high doses showed pronounced hypo-activity, consistent with Wilber (1954) who noted severe depression in bullfrogs following acute sarin exposure. More recently, there have been reports of decreased motor activity in amphibians following OP exposure (Robles-Mendoza et al., 2011). Similar hypo-activity has been reported in tadpoles exposed to OPs (Watson et al., 2014) and these decreases in motor activity were not correlated with AChE inhibition.

In mice that received higher doses of CPO, we noted increased SLUD signs consistent with previous studies (Gaines, 1960; Liu and Pope, 1996); however, we did not observe increased secretions in toads. During the acute lethality studies, toads that received a lethal dose (100 mg/kg) of CPO displayed miosis and extensive parotoid secretions. No brain or liver AChE inhibition was noted in these toads. Chlorpyrifos oxon has been reported to act as direct muscarinic receptor agonist (Huff et al., 1994). It may be that CPO acts directly as a cholinergic agonist at high doses to stimulate the parotoid glands and pupillary sphincters in the absence of AChE inhibition. We also noted erythema in the seat patch region of toads that received higher doses ($\geq$ 62 mg/kg) consistent with previous observations (Wilber 1954).

We predicted that, due to a loss of coordination, intoxicated animals would slide to the bottom of the cage at lower inclines in the tilt test. Chlorpyrifos oxon did not significantly decrease the angle at which mice slid, despite the conspicuous increase in involuntary movements. Surprisingly, intoxicated toads actually had increased stability according to the tilt test. Chlorpyrifos oxon intoxication may depress motor function in toads, preventing the evasive response noted in control animals. The absence of response may allow the toads to adhere passively to the bottom of the cage and thus appear to have greater stability in the tilt test. Future studies should continue to pursue behavioral endpoints for toxicity in amphibians; however, our data suggest that this tilt test is not an appropriate endpoint for OP toxicity in mice or toads.

It should be noted that in mammals the primary cause of mortality is respiratory depression via disruption in the respiratory control center of brain, exacerbated by increased bronchial secretions and loss of diaphragm contractions (De Candole et al.,

1953; Pope, 2006). In contrast, amphibians are not as reliant on constant ventilation and maintain continuous exchange of oxygen through skin, making them resistant to hypoxia (Gargaglioni and Milsom, 2007). Given the lack of AChE depression in toad brain, it is unlikely that disruption of ventilation control occurred, and even if it did, toads would likely be able to withstand hypoxic conditions for several hours without dying from asphyxiation.

In summary, toads were much less sensitive than mice to acute CPO toxicity *in vivo* and did not experience the same degree of enzyme inhibition at equi-toxic doses of CPO. These data suggest that toads are less sensitive to acute toxicity because AChE is not inhibited by CPO *in vivo*. The comparatively low activity and sensitivity of toad CbE suggests that this enzyme does not play a prominent role in the inactivation of CPO. The lack of enzyme inhibition in toads may suggest that toads have a greater ability to detoxify CPO than mouse.

In vitro Comparisons

Acetylcholinesterase in toad brain was significantly less sensitive than mouse brain AChE to inhibition with all inhibitors. These findings are consistent with other *in vitro* studies that demonstrate the relative insensitivity of amphibian AChE to inhibition by OPs and other anticholinesterases (Andersen et al., 1977; Wang and Murphy, 1982; Ferrari et al., 2004). Toads may be less sensitive to acute OP toxicity compared to mice because toad AChE is less sensitive to inhibition. We also evaluated sensitivity of mouse and toad AChE to inhibition by CPO at 26°C to determine if temperature influenced relative sensitivity. Both species had lower basal enzyme activities at 26°C compared to

reactions at 37°C, which is consistent with accepted models of enzyme reactions (i.e., the Q_{10} rule; Wolfenden et al., 1999). Interestingly, the IC_{50} for toad AChE with CPO was greater at 26°C compared to 37°C, while there was no significant difference in the mouse enzyme sensitivity at the different temperatures. As ectotherms, toads may express isoforms of enzymes that are more active at 26°C than 37°C. It may be that such an enzyme binds CPO, and thus increases the IC_{50} for CPO at 26°C relative to 37°C.

There were some kinetic differences between mouse and toad brain AChE. With acetylthiocholine as substrate, there was no difference in V_{max}, but the toad enzyme had a significantly higher k_m compared to mouse enzyme, indicating that the toad enzyme requires a higher concentration of substrate to reach maximal activity. This finding agrees with that of Zahavi et al. (1971), who reported higher K_m of toad AChE with acetylthiocholine compared to several arthropod species and proposed that active site gorge modifications may alter substrate (and inhibitor) access. The comparatively low efficiency of toad AChE reflects phylogenetic differences between mouse and toad; it may also influence the rate at which inhibitors access the active site.

While IC_{50}s are often used to compare sensitivity of enzymes in different tissues, genders and species, etc., it does not necessarily reflect the inherent sensitivity of the enzyme itself. Mortensen et al., (1998) demonstrated that when enzymes are immuno-precipitated from disparate tissues, tissue-related differences in IC_{50}s disappeared, suggesting that the tissue environment around an enzyme could play a role in sensitivity to inhibition. While we have not immuno-precipitated AChE from the tissues in this study, our IC_{50} data suggest that, in its native tissue (environment), toad AChE is far less sensitive than mouse, possibly due to intrinsic detoxification differences.

Toad brain tissue was far more effective at inactivating CPO than mouse brain. This suggests that there may be tissue proteins within the toad brain that serve to protect AChE from inhibition by OPs. These could be enzymes that function as "suicide" inactivators by binding OPs stoichiometrically and thus prevent them from binding AChE. Several authors have suggested that amphibians possess a greater ability than mammals to inactivate OPs following exposure (Edery and Schatzberg-Porath, 1960; Robles-Mendoza et al., 2011); our findings support this hypothesis, i.e., that the toad brain contains proteins that can inactivate CPO.

To characterize any proteins in toad brain that might contribute to local OP inactivation in brain, we screened for OP-sensitive proteins using a fluorescent serine hydrolase probe. There is at least one protein expressed in toad brain that is sensitive to binding by CPO at around 10 nM. Such a protein could contribute to the comparatively high IC_{50} values for OPs with toad AChE; it may also be responsible for the relatively high CPO inactivation noted in toad brain tissue. If this protein is similarly sensitive to OP binding *in viv*o, it may also contribute to the toads' comparatively low acute sensitivity and lack of AChE inhibition following CPO exposure.

In conclusion, our findings demonstrate that the Great Plains toad is less sensitive to acute CPO toxicity than outbred mouse, and that differential sensitivity of AChE may contribute to this species difference. We provide evidence that toad brain tissue is markedly more effective at inactivating CPO compared to mouse brain tissue, which may contribute to differences in sensitivity *in vitro* and *in vivo*. Furthermore, our findings suggest there are additional proteins in toad brain that may contribute to comparatively

higher OP inactivation leading to lesser AChE sensitivity. Identification of this protein may lead to an OP biomarker and a better understanding of OP toxicity in amphibians.

CHAPTER V

SUMMARY AND CONCLUSIONS

- Toads were far less sensitive to acute CPO toxicity compared to mice.
- While mice displayed dose-related inhibition of brain AChE, BChE, MAGL, FAAH and CbE activities, no significant inhibition of these enzymes was observed in toad brain.
- There was no detectable CbE activity in toad brain and low activity in liver relative to mouse, suggesting that in toads, CbE is not a major contributor to OP inactivation.
- After acute CPO exposure, FAAH was significantly inhibited in the liver of both species, possibly representing a new biomarker for OP toxicity in amphibians.
- Toad AChE was markedly less sensitive to *in vitro* inhibition by CPO, paraoxon and naled compared to mouse, which suggests that sensitivity of the target enzyme may contribute to sensitivity *in vivo*.
- Toad brain tissue was substantially more effective at detoxifying CPO than mouse brain. This finding suggests that there are macromolecules in toad brain able to sequester or hydrolyze OPs.
- There is one protein in toad brain sensitive to CPO at 10 nM. This protein may contribute to more effective inactivation of OPs in toad brain.

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Candidate for the Degree of

Master of Science

Thesis: COMPARATIVE IN VIVO AND IN VITRO EFFECTS OF ORGANOPHOSPHORUS ANTICHOLINESTERASES IN THE OUTBRED CD-1 MOUSE AND GREAT PLAINS TOAD

Major Field: Integrative Biology

Biographical:

Personal Data: Born in Houston, Texas, on August 24, 1992, the son of Perry N and Julie S Anderson

Education: Completed the requirements for Master of Science in Integrative Biology at Oklahoma State University, Stillwater, Oklahoma in July, 2017. Completed the requirements for Bachelor of Arts in Biology with a minor in Art at Austin College, Sherman, Texas in May 2015.

Experience: Raised in Sheridan, Wyoming; employed as a house painter, cashier, lifeguard and window repairman during summers; employed by Austin College, Department of Biology as undergraduate research and teaching assistant; employed by Austin College, Department of Art as a sculpture assistant; employed by Oklahoma State University Department of Integrative Biology as a graduate teaching and research assistant.

Professional Memberships: Society of Environmental Toxicology and Chemistry