ORGANOPHOSPHATE INSECTICIDE EXPOSURE AND EFFECTS IN A NON-TARGET BIRD SPECIES

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By

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PREFACE

Chapters 3, 4, and 6 of this thesis have been organized as manuscripts to be submitted for publication in the primary literature, although target journals have yet to be chosen. Chapter 5 of this thesis has been published in the Journal of Wildlife Diseases*. Therefore some repetition of introductory and methodological material is unavoidable.

*Heffernan J, Mineau P, Falk R, and Wickstrom M. 2012. Combined effect of short-term dehydration and sublethal acute oral dicrotophos exposure confounds the diagnosis of anticholinesterase exposure in common quail (Coturnix coturnix) using plasma cholinesterase activity. Journal of Wildlife Diseases 48(3): 695-706.

ABSTRACT

Coturnix quail (*Coturnix coturnix*) were exposed to four different organophosphorus (OP) pesticides (dicrotphos (DCP), ethoprop (ETP), methamidophos (MMP), and naled (NLD)) either by oral gavage or by being enclosed upon a OP-sprayed simulated field of barley for 6 hours in controlled, replicated experiments. The selected OPs varied in solubility. Responses were measured as inhibition of cholinesterase (ChE) activity in plasma and brain. Oral doses were at one-third the estimated median lethal dose while the spray application rates used were expected to produce 60% inhibition of plasma ChE activity 24 hours following removal from the field. Oral doses caused significant inhibition of ChE activity in plasma (33-93%, $p<0.001$) and brain (17-61%, $p<0.001$), confirming that each OP was a ChE inhibitor and bioavailable via the oral route. Recovery from inhibition of ChE activity following oral doses was relatively uniform in plasma (17.6 hours $\leq$ half-life ($t_{1/2}$) $\leq$ 24.5 hours) but slower and more variable in brain (64.8 hours $\leq t_{1/2} \leq$ 128 hours). Responses to field exposures could not be explained by observations of consumption of contaminated feed. Responses to field exposures were inconsistent with those to oral doses with 3 of 4 OPs. The response to field exposures with MMP, the most hydrophilic OP, was generally consistent with the response to oral doses. ChE activity in both tissues was lower following field exposures than following oral doses, significantly so in brain ($p<0.001$), indicating the field dose was larger than the oral dose. Recovery from inhibition of ChE activity in plasma ($t_{1/2}$=17.7 hours) and brain ($t_{1/2}$=125 hours) following simulated field exposures were within the ranges observed following oral doses. The response to field exposures with DCP and NLD, the OPs of intermediate solubility, varied from the response to oral doses. ChE activity in plasma was significantly lower following field exposures than following oral doses ($p \leq 0.002$), while the opposite significant relationship was observed in brain ($p \leq 0.009$). Recovery from

inhibition of ChE activity was prolonged in plasma (DCP, $t_{1/2}$=41.3 hours; NLD, $t_{1/2}$=34.7 hours) and brain (DCP, $t_{1/2}$=34.7 hours) relative to recovery following oral doses. The response to field exposure to ETP, the most lipophilic OP, obviously differed from the response to oral doses. Field exposures produced only moderate inhibition of ChE activity in plasma and no significant inhibition of ChE activity in brain. The conclusion drawn was that the exposure of quail to the OPs on the simulated field was primarily via the dermal route and that quail skin served as a reservoir for storage and delayed release of the OPs, a reservoir effect that increased with increasing lipophilicity of the OP.

Polynomial regression was used to investigate the extent to which inhibition of ChE activity in the brains of quail exposed for 6 hours on the simulated field could be predicted by log of the octanol:water partition coefficients (K_{ow}) of the OPs. Quadratic polynomial curves were significantly fit to brain ChE activity inhibition data at both 24 (r^2=0.888, $p<0.001$) and 72 hours (r^2=0.871, $p<0.001$) post-exposure. These curves indicate that OPs having log K_{ow} in the range of 0 to 1.5 will be those most rapidly absorbed and distributed under field conditions. A similar effort to predict the further inhibition of brain ChE activity in quail associated with the additional exposure to the pesticide spray itself yielded a significant quadratic polynomial curve at 24 hours post-exposure (r^2=0.621, p=0.013). Comments are provided as to the utility and relevance of the findings in ecological risk assessment for pesticide registration.

Quail were exposed to DCP and dehydration in controlled, crossed experiments to determine if dehydration could confound the diagnosis of OP exposure using inhibition of ChE activity in quail tissues. Measures of plasma osmolality (P_{osm}) and hematocrit (Hct) quantified dehydration. DCP exposure caused significant inhibition of ChE activity in brain (38%, $p<0.001$) and plasma (26%, $p<0.001$). Dehydration caused a significant increase in plasma ChE activity

(min. 55%, $p<0.001$). Variation in the change in plasma ChE activity in response to dehydration was significantly and positively correlated with dehydration-induced variation in both the change in P_{osm} ($r^2=0.284$, $p<0.001$) and the change in Hct ($r^2=0.081$, $p=0.018$). The observed correlations suggest plasma ChE activity in quail is not limited to plasma but instead occupies some larger pool of body water. The effects of dehydration on plasma ChE activity masked the inhibitory effects of DCP. Combined dehydration and DCP exposure produced plasma ChE activity that was not significantly different from control values. A method to adjust plasma ChE activities for the confounding effects of dehydration and enable the diagnosis of OP exposure in dehydrated, DCP-exposed quail was developed.

Quail received radio telemetry implants measuring body temperature and heart rate and were given sublethal oral doses of DCP, ETP, MMP, NLD, and vehicle alone. Observations were made before and after exposure and under thermoneutral and cold-stressed conditions. Significant hypothermia and tachycardia were observed under thermoneutral conditions following doses of DCP (1.20ºC, $p<0.001$; 98.4 beats per minute (bpm), $p<0.001$) and ETP (1.21ºC, $p=0.004$; 133 bpm, $p<0.001$). A set of quail exposed to ETP and NLD, but excluded from statistical analyses, failed to thermoregulate under cold-stressed conditions, suggesting that effects on thermoregulation which are sublethal under thermoneutral conditions can become lethal under cold-stressed conditions.

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I first met my supervisor, Dr. Mark Wickstrom, over coffee in the spring of 2006. My first impressions of him in that meeting proved correct over the years that have since passed. He embodies patience, understanding, sympathy, comradery, and good humour. His guidance and assistance over the years has been invaluable. I owe him more than just thanks.

I must also offer thanks to the members of my Committee. Dr. Pierre Mineau was ultimately responsible for the graduate studies opportunity that became this thesis. His criticism and advice on aspects of experimental design, analysis, and writing were at times challenging (rightly so) and have been greatly appreciated. He made me think. Pierre, Ottawa seemed too distant, I wish you had been closer. Dr. Tom Wolf expressed confidence in my abilities while, at times, making it clear that much was expected and the standards are high. If I have laboured long in some aspects of the analysis in this thesis, then Tom deserves much of the credit. Dr. Barry Blakley has been a capable Committee Chair. His advice and occasional prodding have served me well. I am glad he was involved. Dr. Gary Wobeser, the late addition to my Committee, has my gratitude for his willingness to join in on this endeavour at a time when his retirement approached. I hope there has been some reward and satisfaction for Gary in doing so.

This list of others who had a hand (quite literally, for many) in this work is lengthy and I hope I have not forgotten any individuals. Brian Caldwell of Agriculture and Agri-Food Canada played a key role in this work. He was responsible for the pesticide spray applications in the simulated field exposures upon which this thesis is founded. His substantial investment in this effort was both mental and physical. It could not have been completed without him. Ramona Falk was largely responsible for my first ‘eureka moment’ and the resulting publication. She helped to develop the experimental design and performed the bulk of the experimental work

described in Chapter 5 of this thesis. Both Laura McKenny and Tracy MacDonald deserve thanks for their time spent working in the radio telemetry experiment. Laura set up the hardware and software and tested the system on rodents while Tracy spent many hours in an environmental chamber handling quail and collecting experimental data. Altina Wickstrom was invaluable in figuring out how best to adapt the rodent radio telemetry devices for use in quail. She also occasionally helped with the collection of tissue samples. Thanks also go to Tressie Wickstrom for similar help. Indeed the entire Wickstrom family deserves recognition for their assistance.

Dr. Paul Jones has my gratitude for his efforts in residue analyses (efforts which continued beyond the thesis defence). Paul, I offer my apologies for imposing dicrotophos and ethoprop upon you. They are certainly not the well-behaved persistent organic pollutants to which you are accustomed. I asked a great deal of you for very little in return.

My own family deserves recognition for their unwavering support in the years consumed by this work. The financial assistance of my mother, Margaret Heffernan, and brother and sister-in-law, Dan Heffernan and Randi Arnot, in particular has been extremely important. Their investment allowed me to focus exclusively on this thesis over the last few months of work. Thank you all!

I have made many, many friends while in the Toxicology Graduate Studies Program and they all deserve thanks for helping to preserve my sanity and maintain my mental health. When I consider all the poker played with Jonathan Naile, David Vardy, Jake Ouellet, Jeff Thomsen, Eric Higley, Shawn Beitel, Jon Doering, Brett Tendler, and David Saunders, among others, I believe I have probably broken even monetarily while accumulating countless smiles, laughs, jokes, pats on the back, and friendly barbs. Others who have shared with me the fun and frustration of grad school and life at the Toxicology Centre and so deserve recognition include

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A very special thank you is extended to Ashley James. If anyone had suggested to me at the outset of this work that I, the youngest of five siblings, would learn what it is to be an older brother while completing my degree, I think I would have doubted them greatly. But you, "little sister", are an absolute gem of a human being and I have come to think of you as family! Sharing an office with you for a few years proved to be an incredible and entirely unexpected blessing. You deserve more thanks than you know.

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Lastly, there are innumerable scientists, researchers, and statisticians whose publications have served me. Many have been cited in this work. Others have simply been informative and inspiring. I am standing on your shoulders. Thank you.

In Memoriam

William John Heffernan

I had hoped that my father would live to see the day I completed my thesis. He would have enjoyed celebrating the accomplishment with me as he was always a student at heart himself. His clarity of thought, pension for logic, and ethos made him a good academic role model. His unwavering support of my academic efforts was greatly appreciated.

Brayden Dean Houdek
Gregory George Lydon Alan Dubetz
Gary Roy Anthony Bortolotti

In the years it has taken me to restructure my life and future, these friends (Brayden and Greg) and mentor (Gary) have lost theirs. They are all greatly missed.

I suggest to you all that much insight has been gained in the pursuit of fine beer.

"When a quantity is measured with all possible precision many times in succession, the figures expressing the results do not absolutely agree, and even when the average of results, which differ but little, is taken, we have no means of knowing that we have obtained an actually true result, and the limits of our powers are that we can place greater odds in our favour that the results obtained do not differ more than a certain amount from the truth."

"Statistical examination in each case may help much, but no statistical methods will ever replace thought as a way of avoiding pitfalls, though they may help us to bridge them."

Both above by William S. "Student" Gosset
Brewer, Messrs Authur Guinness Son and Co., Ltd., circa 1904

"…only naughty brewers take *n* so small that the difference is not of the order of the probable error!"

Karl Pearson in writing to Gossett, 1912

All quotes above in
Pearson, Egon S. 1939. "Student" as statistician. Biometrika 30: 210-250.

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LIST OF ABBREVIATIONS

Δ	change in
η^2	eta-squared
η_p^2	partial eta-squared
AAFC	Agriculture and Agri-Food Canada
AChE	acetylcholinesterase
ANOVA	analysis of variance
AThCh	acetylthiocholine iodide
BChE	butyrylcholinesterase
C	Celcius
ChE	cholinesterase
CM	carbamate
CV	coefficient of variation
DCP	dicrotophos
DF	dilution factor
DTI	dermal toxicity index
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
ECV	extracellular fluid volume
EEC	estimated environmental concentration
EPA	Environmental Protection Agency
ERA	ecological risk assessment
ETP	ethoprop
FC	final concentration
FD	field plus dietary
FIFRA	Federal Insecticide, Fungicide and Rodenticide Act
FO	field only
FD	field plus diet
FS	field plus spray
H	hydrogen
Hct	hematocrit
HLC	Henry's law constant
HR_c	heart rate under cold-stress conditions
HR_t	heart rate under thermoneutral conditions
HSD	honest significant differences
iso-OMPA	tetraisopropyl pyrophosphoramide
J_{max}	maximum skin permeation rate
k	rate constant
K_{ow}	octanol:water partition coefficient
K_p	skin permeability

LC_{50}	median lethal concentration
LD_{50}	median lethal oral dose
M	molar
MMP	methamidophos
MV	molecular volume
MW	molecular weight
n	sample size
NC	negative control
NCT	nicotine
NLD	naled
NSAIDs	non-steroidal anti-inflammatory drugs
NTG	nitroglycerine
OC	organochlorine
OLS	ordinary least squares
OP	organophosphorus or organophosphate
PB	physiologically-based
PBS	phosphate-buffered saline
PD	pharmacodynamic
PK	pharmacokinetic
P_{osm}	plasma osmolality
PC	positive control
PV	plasma volume
QM	quotient method
QSAR	quantitative structure-activity relationship
QSPR	quantitative structure-permeability relationship
RTO	regression through the origin
RQ	risk quotient
SC	stratum corneum
SD	standard deviation
SE	standard error
SS	sum of squares
Std	standard
TBW	total body water
T_c	body temperature under cold-stress conditions
TChE	total cholinesterase
T_t	body temperature under thermoneutral conditions
TP	toxic potential
UK	United Kingdom
Unk	unknown
US, USA	United States of America

VE	viable epidermis
v/v	volume per volume

Chapter 1

1.0 INTRODUCTION

1.1 General

This thesis is part of a larger terrestrial effects research project developed by Drs. Pierre Mineau and John Elliot, Research Scientists with Environment Canada. The focus of their project was on developing means to quantify the exposure of and mitigate impacts on birds and species at risk resulting from pesticide use in agricultural lands. The project consisted of three distinct subprojects, of which this thesis describes one. The two related subprojects examined i) the impacts of selected pesticides, including chlorpyrifos, on terrestrial wildlife on intensely farmed lands in southwestern British Columbia and ii) the impact of rodenticides on species listed in the Species at Risk Act and occurring within the Okanagan valley of British Columbia. The subproject described in this thesis was developed to compare the contributions of various routes of exposure (i.e. food-borne, dermal, other) to the effects of pesticide sprays on non-target birds under simulated field conditions and to establish the extent to which the physicochemical properties of the active ingredients in the sprays influence their contributions to effects of the various routes of exposure and then to construct a predictive exposure model. The intent was to produce an exposure model of utility to regulators in Canada and abroad in estimating the risks of pesticide sprays to birds from various routes of exposure.

A set of controlled, replicated spray exposure experiments was completed on simulated cereal crops with a model non-target game bird. All pesticides were organophosphorus (OP) compounds having a common anticholinesterase mechanism of action but varying in physicochemical properties.

1.2 Collaborators and Facilities

Research efforts were supervised by Dr. Mark Wickstrom of the Toxicology Centre, University of Saskatchewan. All spray exposures were conducted within an experimental spray room at Agriculture and Agri-Food Canada (AAFC) at the University of Saskatchewan under the supervision of Dr. Tom Wolf. Spray applications at AAFC were performed by Brian Caldwell, a licensed applicator. All plant material used in the experiments was grown under greenhouse conditions at AAFC while soil used on the simulated field was obtained from nearby AAFC lands. Initial effects assays were conducted by Prairie Diagnostic Services at the Western College of Veterinary Medicine (WCVM), University of Saskatchewan under the supervision of Dr. Barry Blakley. Later, all experimental effects assays were completed by the author in the Biochemical Toxicology Laboratory in the Toxicology Centre, University of Saskatchewan. All experimental birds were housed at the Animal Care Unit in the WCVM, University of Saskatchewan.

1.3 Toxicants

A commercial formulation of chlorpyrifos (Lorsban™ 4E, chlorpyrifos 480 g/L, Dow AgroSciences Canada Inc.), an organophosphorothioate compound requiring bioactivation *in vivo*, was used in initial pilot studies conducted prior to the experiments because it was being the compound of interest in related research by Environment Canada. It was later replaced by a set of commercial formulations of directly toxic organophosphate compounds to avoid any potentially confounding effect related to the need for *in vivo* bioactivation. The commercial formulations selected for the bulk of the research were BIDRIN® 8 (dicrotophos (DCP) 82.0%, Amvac Chemical Corporation), MOCAP® EC (ethoprop (ETP) 69.6%, Bayer CropScience Inc.),

MONITOR® 480 (methamidophos (MMP) 480 g/L, Bayer CropScience Inc.), and DIBROM® (naled (NLD) 864 g/L, United Agri Produces Canada Inc.). These organophosphate compounds were selected for their common acute toxicity and mechanism of action and their diversity in physicochemical properties.

1.4 Model Species

Two different model species were used in this research; northern bobwhite (*Colinus virginianus*) and coturnix quail (*Coturnix coturnix*). The northern bobwhite (Silver Maple Game Farm, Millbank, Ontario, Canada) was originally selected as the model species as it is a game bird that is commonly used in toxicology tests and, as such, there are data available on the toxicity of numerous compounds to this species. Unfortunately, the supply of this species for experimental purposes could not be assured and its use was limited to some initial pilot studies. The coturnix quail (Fircrest Farms Ltd., Langley, British Columbia, Canada) was subsequently selected as the model species for the bulk of the pilot studies and all experiments as its supply was reliable. Information on the toxicity of compounds to this species and its physiology was obtained from primary literature using this species and the closely related Japanese quail (*Coturnix japonica*).

1.5 Research Goals

There are several components to the thesis research program. The primary focus of the thesis is to quantify the extent to which different routes of exposure contribute to the toxicity of OP pesticides in birds under simulated field conditions. Closely related to this effort is the development of a predictive avian-OP spray exposure model that requires only the

physicochemical properties for the OPs, oral toxicity, and field application rates as input. Secondary effort is focused on the feasibility of using radio telemetry implants to investigate how OP exposure influences cardiac and thermoregulatory function in birds under different temperature regimes. The final component of the thesis is an investigation of whether or not short term dehydration confounds the diagnosis of anticholinesterase exposure in birds. Each of these components is intended to address an existing need.

The current avian ecological risk assessment screening level model has changed little since its development in the 1970s (Fite 1994). The fundamental assumption of this model is that consumption of contaminated food is the primary source of exposure of birds to pesticides (Fite 1994). The realism of this paradigm has been questioned (Driver et al. 1991, Matz et al. 1998, Mineau 2002, Vyas et al. 2006). Further, the extensive field investigations required by higher tier risk assessments to validate the presumption of 'no effect' of pesticide use on a compound by compound basis are cost prohibitive (Mineau 2002). Hence, there is an existing need for predictive exposure models that can both estimate the toxicity from multiple exposure routes and be applied broadly to classes of related compounds. Controlled, replicated exposures of birds to four different OP pesticide sprays are proposed under simulated field conditions to determine the relative contributions of different exposure routes. Results of the experiment will be used in building a predictive exposure model.

Numerous sublethal effects of OP pesticides are known, including effects on avian physiology (Rattner and Fairbrother 1991). It has been suggested that sublethal effects can contribute to the decline of bird populations (Walker 2003). However, little empirical evidence exists to explain how the sublethal effects of pesticides in birds can influence population parameters (Heinz 1989). Substantial investigation and the application of novel tools to the

problem are both required if this matter is to be better understood (Heinz 1989). The use of radio telemetry implants are proposed to investigate how sublethal OP exposure affects heart rate and thermoregulatory function in birds under different temperature regimes and to demonstrate the feasibility of using these techniques with birds.

The number of methods used to diagnose anticholinesterase exposure is vastly exceeded by the number of factors that can confound such a diagnosis. Diagnosis of anticholinesterase exposure in birds typically relies on measurement of cholinesterase activity in brain, plasma, or both tissues (Rattner and Fairbrother 1991). Brain, the tissue arguably most useful in the diagnosis of anticholinesterase exposure (Rattner and Fairbrother 1991), cannot be sampled without killing the subject. Plasma can be sampled with little harm (Thompson 1991) and is more sensitive to anticholinesterase exposure than brain (Ludke et al. 1975). Unfortunately the utility of plasma in the diagnosis of anticholinesterase exposure is limited by the high variability of cholinesterase activity in plasma (Thompson 1999). This variability in plasma is due to several factors. Within a species, brain ChE activity may vary with age (Grue and Hunter, 1984; Custer and Ohlendorf, 1989) and nutrition (Rattner, 1982) while that in plasma may vary with sex (Ludke et al., 1975; Hill, 1989; Maul and Farris, 2004), age (Ludke et al., 1975; Fairbrother et al., 1990), hormone status (Fairbrother et al., 1989), time of day (Cobos et al., 2010), and season (Hill and Murray, 1987). Such factors may be problematic in the diagnosis of anticholinesterase exposure and hamper efforts to investigate and monitor the effects of pesticides on birds. The affect of dehydration on cholinesterase activity in birds has not been studied and is a potential confounding factor in the diagnosis of anticholinesterase exposure. A controlled, replicated experiment is proposed to examine how dehydration affects brain and plasma cholinesterase activity in birds.

The several working hypotheses of this proposal are that: 1) oral, dermal and other routes of exposure all contribute to OP toxicity in birds in the field; 2) the contribution of different routes of exposure to total toxicity in OP exposures can be explained by the physicochemical properties of the OPs; 3) sublethal exposure of OPs to birds disrupts normal cardiac and thermoregulatory functions in birds; and 4) short term dehydration can confound the use of ChE inhibition to diagnose OP exposure.

This research is specifically intended to:

i. Determine the contributions of various routes of exposure to total toxicity in birds from spray applications of a set of OP pesticides of varying physicochemical properties under simulated field conditions;
ii. Develop an exposure model for predicting the contributions of the oral, dermal, and potentially other routes of exposure to total toxicity of OP pesticide sprays in birds based upon the physicochemical properties of the OP itself and its application rate;
iii. Determine if short term dehydration of birds confounds the diagnosis of OP-exposure using measures of ChE activity in brain or plasma; and
iv. Determine if sublethal oral exposure to OPs adversely effects cardiac and thermoregulatory function in birds under different temperature regimes (18°C and 4°C) using radio telemetry implants.

1.6 Thesis Structure

Chapter 2 is a literature review describing the current state of knowledge relating to the exposure of and effects upon birds resulting from the use of organophosphorus pesticides on agricultural lands, including comments on regulatory practice and modeling as they relate to

assessing the risk to birds from use of these compounds. Chapter 3 describes the pesticide spray exposure experiment on a simulated cereal crop while Chapter 4 describes the predictive exposure model developed from observations presented in Chapter 3. Chapter 5 describes the investigation into whether dehydration can confound the diagnosis of anticholinesterase pesticide exposure in birds. Chapter 6 describes the investigation into the sublethal effects of anticholinesterase exposure in birds on thermoregulation and cardiac function. Chapter 7 presents comments on, and arguments for, the further investigation of how different routes of exposure contribute to the toxicity of anticholinesterase pesticides in birds on agricultural lands.

Chapter 2

2.0 LITERATURE REVIEW

2.1 Toxicity of Organophosphate Pesticides to Non-target Organisms

2.1.1 Cholinesterase Inhibition as a Biomarker of Exposure and Effect

Aldridge and Davison (1953) first described the mechanism of action of several OP compounds in inhibiting the activity of cholinesterase. OP compounds of the general formula $(R)_2(X)PO$ were found to produce identical inhibited cholinesterase for constant R groups while the X group was varied (Aldridge and Davison 1953). The X group was labile in hydrolysis of the OP compounds and phosphorylation was confirmed as the mechanism of cholinesterase (ChE) inhibition (Aldridge and Davison 1953). This mechanism of action is common in the several uses of OP compounds including therapy, nerve gases, and, of course, pesticides (Pope et al. 2005).

Fukuto (1990) provided a concise description of the mechanism of action of the OP pesticides. Here the target is acetylcholinesterase (AChE), an enzyme essential in nervous transmission and common to many animals including humans, birds, and insects. In its endogenous function, AChE is acetylated at a serine hydroxyl moiety by the neurotransmitter acetylcholine (ACh) while the neurotransmitter is degraded to choline. The acetylated AChE then rapidly breaks down to acetic acid and the original enzyme while the choline is recycled in the production of new ACh. In the case of inhibition, a reaction occurs between the AChE and the OP pesticide molecule in which AChE is phosphorylated at the same serine hydroxyl moiety while the X group leaves the reaction. The phosphorylated AChE is very stable and inhibited. A local increase in ACh results when AChE inhibition is sufficiently large.

In vertebrates, the interaction of ACh and AChE is critical in nervous transmission at cholinergic, synaptic, and neuromuscular junctions (Pope et al. 2005). The inhibition of AChE and resulting build up of ACh in these junctions produce several effects described generally as cholinergic toxicity. In mammals, stimulation of secretory organs and smooth muscle tissues produce recognizable symptoms of cholinergic toxicity including salivation, lacrimation, urination, and defecation. Other symptoms include constriction of the iris, involuntary movements, paralysis, and cardiac arrhythmia. Mortality is typically due to respiratory failure from excessive secretions within the airways and respiratory muscle paralysis. Symptoms and lethality in birds are similar.

Inhibition of cholinesterase (ChE) activity in tissues of birds is routinely used to diagnose exposure to OP and carbamate pesticides (Thompson 1999). Avian ChE activity is commonly measured in brain (Fleming and Bradbury 1981, DeWeese et al. 1983, Niethammer and Baskett 1983, McEwen et al. 1986, Robinson et al. 1988, Rendon-von Osten et al. 2005) and plasma (Fleming and Bradbury 1981, Fairbrother et al. 1989, McInnes et al. 1996, Wilson et al. 2001, Maul and Farris 2005, Fildes et al. 2006). Brain is the preferred tissue for investigating anticholinesterase exposure in bird carcasses because of the preponderance of ChE activity in the organ (Rattner and Fairbrother 1991) and the persistence of inhibition in the brain (Westlake et al. 1981). Plasma is commonly used to assess exposure in live birds as sampling is non-lethal (Thompson 1991, Fossi et al. 1992), it is sensitive to anticholinesterase exposure (Ludke et al. 1975), and because repeated measures in plasma provide the ability to perceive changes within an individual bird (Fairbrother et al. 1989). The utility of inhibition of plasma ChE activity as a measure of exposure is diminished by the high degree of variability in normal ChE activity (Thompson 1999) and the short duration of its inhibition from anticholinesterase exposure

(Westlake et al. 1981). Diagnosis of anticholinesterase exposure in birds may also be made with the reactivation of ChE activity in these tissues (Stansley 1993, Smith et al. 1995, Maul and Farris 2005).

Greater than 20% and >50% inhibition of ChE in brain are accepted as being diagnostic of sublethal and lethal exposures to anticholinesterases, respectively (Ludke et al. 1975). Although no similar diagnostic thresholds exist for plasma ChE activity, correlations have been observed between the peak inhibition of ChE activity in brain and plasma over a range of OP acute oral doses (Fossi et al. 1992).

Several confounding factors must be considered in making a diagnosis of exposure with either brain or plasma ChE inhibition (Rattner and Fairbrother 1991). Within a species, brain ChE activity may vary with age (Grue and Hunter 1984; Custer and Ohlendorf 1989) and nutrition (Rattner 1982), while that in plasma may vary with sex (Ludke et al. 1975; Hill 1989; Maul and Farris 2004), age (Ludke et al. 1975; Fairbrother et al. 1990), hormone status (Fairbrother et al. 1989), time of day (Cobos et al. 2010), and season (Hill and Murray 1987). However, the effect of hydration state on ChE activity in the tissues of birds appears to have been little studied, apparently limited to waterfowl possessing salt glands (Herin et al. 1978, Rattner et al. 1983). The effects of anticholinesterase exposure and an osmoregulatory challenge on ChE activity has been studied in mallards (*Anas platyrhynchos*) (Herin et al. 1978). Salt gland and brain acetylcholinesterase (AChE) activity in mallard ducklings were significantly inhibited by dietary chlorpyrifos exposure while the effects of salt loading in drinking water on these endpoints was not significant (Herin et al. 1978). Similarly, ingestion of fenthion by American black ducks (*Anas rubripes*) caused significant inhibition of both brain and plasma ChE activity, while the effect of saline drinking water on these endpoints was not significant (Rattner et al.

1983). Further, no change from normal brain AChE activity was found in the carcasses of waterfowl that died of salt toxicosis in North Dakota (Windingstad et al. 1987) and New Mexico (Meteyer et al. 1997).

It seems obvious that wild birds experience periods of water shortage and abundance due to environmental conditions and so can become ephemerally dehydrated. Dehydration of birds can also be the consequence of disease (Joyner et al. 2006, Neimanis et al. 2007) and toxicity resulting from exposure to environmental pollutants (Wynne and Stringfield 2007). Therefore if dehydration is a confounder in the diagnosis of anticholinesterase exposure, then it is a confounder that may frequently be problematic.

2.1.2 Physiological and Organismal Responses

Direct mortality is not the only adverse effect of anticholinesterase exposure in birds (Grue et al. 1991, Pope et al. 2005). Outward symptoms in birds of sublethal cholinergic toxicity include ataxia, blindness, convulsions, dyspnea, hyperexcitability, incoordination, lethargy, piloerection, slurred vocalizations, and tremors, among others (Grue et al. 1991). Sublethal effects on behavior can impair survival and reproduction (Grue et al. 1991). Behavioural effects include altered activity patterns, reduced food and water intake, aggression, and adverse effects in nest defense and parental care (Grue et al. 1991). There are also adverse effects on thermoregulation and cardiac function (Grue et al. 1991).

Although obviously related, more is probably known about how thermal stress affects OP toxicity in birds than about how OP toxicity affects the ability of birds to thermoregulate. Thermal stress increased the lethality of OP pesticides in American kestrels (*Falco sparverius*) (Rattner and Franson 1984), mallard ducklings (Fleming et al. 1985), Japanese quail (*Coturnix*

japonica)(Rattner et al. 1987), and northern bobwhite (Maguire and Williams 1987). Rattner and Franson (1984) exposed American kestrels to graded doses of methyl parathion by post-oral gavage. Kestrels were subsequently exposed to thermoneutral (+22°C) or cold-stressed (-5°C) conditions for 10 hours. Kestrels exhibited pronounced hypothermia in association with 50% or greater depression of plasma and brain ChE activity. The magnitude and duration of the hypothermic response increased with dose. Three of the 5 kestrels exposed to 2.25 mg/kg methyl parathion and placed in cold-stressed conditions died whereas there was no mortality in a similar group under thermoneutral conditions. Fleming et al. (1985) exposed day old mallard ducklings to graded dietary concentrations of temephos for 7 days. Ducklings in the dietary exposures were housed in either heated (39–41°C) or non-heated brooders (10-18°C). Duckling mortality was greater in the non-heated brooders than in the heated brooders at all dietary concentrations of temephos. High duckling mortality was observed only in the unheated brooders at the highest dietary concentration of temephos. Rattner et al. (1987) conducted median lethal dose experiments with ethyl parathion and Japanese quail exposed acutely or chronically to the elevated, thermoneutral, or reduced temperatures of 37, 26, or 4°C, respectively. The median lethal dose calculated for ethyl parathion was significantly lower for quail exposed chronically to either elevated or reduced temperatures relative to the thermoneutral group. Acute heat exposure also resulted in a significantly lower median lethal dose of ethyl parathion in quail. Acute cold exposure resulted in a non-significant reduction in the medial lethal dose. Maguire and Williams (1987) exposed juvenile northern bobwhite to chlorpyrifos in a standard avian dietary test under constant cold-stressed conditions. Dietary chlorpyrifos concentrations varied from 100 to 1000 ppm while ambient temperatures ranged from 27.5 to 35.0°C. A dose-response relationship was apparent between chlorpyrifos concentration and the magnitude of observed hypothermia.

Survival of juvenile bobwhite decreased significantly under cold-stressed conditions. The estimated median lethal dietary chlorpyrifos concentrations were 531, 497, 345, and 283 ppm at 35.0, 32.5, 30.0, and 27.5°C, respectively.

The effects of OP pesticides on cardiac function in birds have been investigated (McFarland and Lacy 1968), although not extensively. There are more abundant data available in mammals (Gordon and Padnos 2000, Smith and Gordon 2005, Çetin et al. 2007). McFarland and Lacy (1968) observed tachycardia and heart block in Japanese quail, mallards and northern pintails (*Anas acuta*) injected intraveinously with parathion at 3.9-5.7, 0.61-1.70 and 1.57-2.02 mg/kg, respectively. Gordon and Padnos (2000) observed significant bradycardia in rats exposed to chlorpyrifos at 25 mg/kg. In contrast, Smith and Gordon (2005) observed significant tachycardia in rats exposed to chlorpyrifos at the same dose. Çetin et al. (2007) observed significant bradycardia, decrease in cardiac output, and other measures of cardiac dysfunction in rabbits exposed to 375 ppm chlorpyrifos in drinking water.

2.2 Ecological Risk Assessment for Pesticide Registration

2.2.1 The Regulatory Framework in North America

The regulatory frameworks governing pesticide use in Canada and the United States (USA) are similar. Pesticide products must be registered with the relevant agencies in each nation for their sale and use to be legal. Pesticide registration is at once an administrative, legal, and scientific process, wherein the risks to human health and the environment are evaluated. Registration results in strict limits and conditions for the production, storage, use, and disposal of pesticides. Pesticide products are re-evaluated on a regular basis.

In Canada, the Pesticide Management Regulatory Agency (PMRA), a branch of Health Canada, is responsible for pesticide registration. The PMRA is granted the authority for pesticide registration under Canada's *Pest Control Products Act*. In the USA, it is the Environmental Protection Agency (EPA) that is responsible for pesticide registration under the *Federal Insecticide, Fungicide, and Rodenticide Act* (FIFRA).

2.2.2 The Current Paradigm in the Assessment of Pesticide Risks

The current avian pesticide assessment model was developed for the EPA in 1975 and has changed little since (Fite 1994). Briefly, it is a tiered process utilizing a series of tests beginning with laboratory tests and proceeding to field tests when unacceptable hazards are identified (Fite 1994).

The initial screening level ecological risk assessment (ERA) methodology adopted by the United States EPA for registration of pesticides under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) is based on a quotient method (QM), in which a risk quotient (RQ) results from the ratio of an estimate of exposure over a reference measure of toxicity (Urban and Cook 1986). The resulting RQs are compared to established screening criteria (Fite 1994). The model exposure estimates are based on the assumption that contaminated food items are the sole source of exposure (Fite 1994). Residues on vegetative food items are estimated based on the data of Hoerger and Kenaga (1972). Residues on insect food items are estimated based on the data of Kenaga (1973).

Toxicity thresholds are established using standardized laboratory acute oral and dietary tests using single species (Fite 1994). The species used are either northern bobwhite or mallards. Toxicity tests estimate the quantity of pesticide required to produce an effect in the species.

When mortality is the endpoint, the toxicity threshold is presented as a median lethal dose (LD_{50}) or median lethal concentration (LC_{50}) in feed. Screening criteria serve to separate pesticides into three categories; those with acceptable hazards, those with unacceptable hazards that may be mitigated by product labeling, and those with unacceptable hazards. It is presumed that the screening criteria provide a margin of safety for pesticides with acceptable hazards such that there will be minimal impact on birds. Several assumptions and uncertainties inherent in the model. The fundamental assumption of this model is that consumption of contaminated food is the primary source of exposure of birds to pesticides. Further, the model fails to account for within-species and between-species differences in sensitivities to pesticides. Differences between laboratory and field conditions are not examined for pesticides with acceptable hazards, even though there are bound to be differences in one or more of duration of exposure, environment, behavior, cohort etc. Further, as pesticides with acceptable hazards are never field tested, the margins of safety inherent in the screening criteria for categorizing pesticides are rarely examined.

2.2.3 Criticism of Ecological Risk Assessment Practice

Ecological risk assessment (ERA) has been adopted widely in a variety of regulatory settings (Suter II 2008). Further, ERA practice has been adapted to suit the policy and regulatory needs within these settings (Suter II 2008). Despite its widespread use, there exists polarity in opinion regarding the utility and practice of ERA (Power and Adams 1997). Proponents argue it is a valuable tool integrating science with decision making (Barnthouse 2008). Detractors argue ERA practice routinely oversimplifies ecological problems and fails to validate its predictions (Power and Adams 1997). Both proponents and detractors recognize that ERA is an evolving

practice and that it can and will be improved (Barnthouse 2008, Kapustka 2008). Challenges facing ERA practice include the need for validation (Power and Adams 1997) and improved methods to deal both with dynamic ecological processes and with potential biases of and limitations imposed upon practitioners (Kapustka 2008).

Much of the criticism directed at ERA generally is also directed specifically towards the QM in use in screening level ERA in pesticide registration. Bascietto et al. (1990) describe several weaknesses in the QM, including failing to characterize dose-response relationships, failing to account for differences between laboratory and field conditions, being of unknown reliability, and providing no means to quantify uncertainty. Further, there exist abundant records of avian mortality resulting from pesticide use (Fleischli et al. 2004) and an investigation of one such incident concluded that the use of some OPs may adversely impact sage grouse (*Centrocercus urophasianus*) populations (Blus et al. 1989).

Reports of bird kills from OP pesticide poisoning have been made worldwide (Fleischli et al. 2004), but comprehensive reviews and analyses of these reports are uncommon (Greig-Smith 1994, Mineau 2002, Fleischli et al. 2004). A review of United States Geological Survey National Wildlife Health Center voluntary reports of avian mortality caused by anticholinesterase pesticides in the United States between 1980 and 2000 found that OP compounds were responsible for 249 of the 335 reported incidents of confirmed or suspected poisoning (Fleischli et al. 2004). Reports were received from 42 different states (Fleischli et al. 2004). Total reported avian mortality from confirmed or suspected anticholinesterase poisoning, both OPs and carbamates, was 8877 birds of 103 different species in 12 different Orders (Fleischli et al. 2004). The three OPs, famphur, diazinon, and fenthion, were implicated in 116 of these incidents having a total mortality of at least 1984 birds (Fleischli et al. 2004). Fifteen other OPs were also

involved in these incidents of avian mortality (Fleischli et al. 2004). Greig-Smith (1994) lists incidents of bird poisoning in England and Wales as recorded in the United Kingdom Wildlife Incident Investigation Scheme during the period of 1980 to 1989. Only incidents in which more than 50 birds were found dead and the cause was attributed to the approved use of pesticides were recorded by the scheme (Greig-Smith 1994). Eight different OPs were involved in 12 incidents of mortality of more than 1000 birds in 12 species (Greig-Smith 1994). Both Fleischli et al. (2004) and Greig-Smith (1994) noted that the incident reports they reviewed underestimate the mortality associated with a particular incident and fail to identify all occurrences of bird mortality from pesticide use. Vyas (1999) described several factors associated with pesticide-caused mortality that explain why incident reports underestimate the scope and magnitude of avian mortality from pesticides. Pesticide-caused bird mortality must first occur, then be observed, then be reported, and finally be confirmed (Vyas 1999). As failures in observation, reporting, and confirmation are known, confirmed reports of pesticide poisoning of birds strongly imply that such incidents may be common (Vyas 1999).

Blus et al. (1989) investigated reports of sage grouse die-offs in Idaho from 1981 by radio-tagging grouse in the summers of 1985 and 1986. A total of 82 seemingly healthy sage grouse within a larger local population were tagged in the two summers. Fifteen of the 82 tagged grouse subsequently entered fields sprayed with either dimethoate or methamidophos applied at maximum label rates. Mortality rates in tagged grouse were 5 and 16% in 1985 and 1986, respectively. Other observations of the local sage grouse population revealed >30% mortality among grouse occupying an alfalfa field at the time it was sprayed with dimethoate. Sixty-three carcasses were recovered from this alfalfa field and the brains of 43 of those exhibited >50% inhibition of ChE activity. Further, tagging and monitoring of 31 intoxicated grouse captured on

a dimethoate-sprayed alfalfa field revealed a greater than 60% mortality rate in these grouse. They concluded that some OPs may adversely affect sage grouse populations.

Efforts to estimate the actual avian mortality associated with the use of a specific pesticide based upon incident reports have been few because data are generally lacking for single pesticides (Mineau 2005). Mineau examined data from the US EPA's special review of the granular formulation of carbofuran, an anticholinesterase pesticide of the carbamate variety, and estimated bird losses. This product was chosen because the EPA special review had generated the best available data set for such an exercise. The estimated worst case scenario for granular carbofuran on corn was a kill rate in the range of 3 to 16 birds per hectare. There were approximately 5.7 million hectares of granular carbofuran-treated corn in the US in 1978. By extrapolation, this single formulation of a single anticholinesterase pesticide on a single crop may therefore have been responsible for 17 to 91 million bird deaths in 1 year. Only a few reports of bird kills resulting from granular carbofuran use preceded the EPA special review leading to the conclusion that the absence of carcasses is not necessarily an indication of safe pesticide use.

Lethal risks to birds have also been estimated based upon avian mortality data sets for all anticholinesterase pesticides applied as sprays (Mineau 2002, Mineau and Whiteside 2006). These efforts were used to compare products and develop predictive models (Mineau 2002) and to describe how lethal risks to birds have varied over time with crop, state, and active ingredient in the US (Mineau and Whiteside 2006). The models of Mineau (2002) were applied to data sets for specific pesticide-crop combinations on a state by state basis in the US from 1991 to 2003 (Mineau and Whiteside 2006). Lethal risks to birds in the US generally declined from 1991 to 2003 in association with the introduction of new pesticides of novel chemistry (Mineau and

Whiteside 2006). No similar trend was observed in association with anticholinesterase pesticides alone (Mineau and Whiteside 2006).

2.2.4 Validation of Ecological Risk Assessment Practice

There are only a few published studies that have attempted to field validate the QM used in screening level ERA for pesticide registration, some with mammals (Edge et al. 1996, Wang et al. 1999, Edge and Schauber 2000) and others with birds (Matz et al. 1998, Vyas et al. 2006). The publications of Edge et al. (1996), Wang et al. (1999), and Edge and Schauber (2000) are among several published by a group conducting QM validation work with small rodents, typically the grey-tailed vole (*Microtus canicaudus*), in pen studies with azinphos-methyl (AM). Population-level impacts (i.e. mortality) were estimated using mark-recapture methods (Edge et al. 1996, Wang et al. 1999, Edge and Schauber 2000). Edge et al. (1996) observed significant dose-dependent declines in population size and survival of both sexes in vole populations exposed to AM sprayed on alfalfa in pens. These effects were of short duration, suggesting a compensatory response within the population (Edge et al. 1996). They concluded that the QM accurately predicted the risks to voles (Edge et al. 1996). Wang et al. (1999) conducted similar experiments with a vole population on dense pasture grasses in pens and found no effects on population parameters. The QM overestimated the hazards to the vole population under these conditions and this was attributed to the dense pasture grass canopy having sheltered the vole population from exposure to AM (Wang et al. 1999). Edge and Schauber (2000) studied the impacts of various application rates of AM on alfalfa to both voles and deer mice (*Peromyscus maniculatus*) in pens. They were able to predict of mortality in each species as they had access to data from dietary toxicity studies with AM for both species (Edge and Schauber 2000). They

found that their estimates of mortality based upon mark-recapture data correlated with both the QM predictions of risk and with predictions of mortality derived from dietary study data and estimates of residues on the field (Edge and Schauber 2000). The extent to which such findings with resident, generally r-selected rodents are applicable to migratory, more k-selected birds typical on agricultural lands in temperate North America, is highly debatable. Attempts to validate the QM using birds have, to date, relied on measures of impacts to individuals (Matz et al. 1998, Vyas et al. 2006), not populations.

Comparisons of field and laboratory toxicity tests using OP pesticides and birds found that the EPA ecological risk assessment quotient method underestimates the risks to birds exposed to OPs in the field (Matz et al. 1998, Vyas et al. 2006). Matz et al. (1998) compared the results of a controlled field study with a standard laboratory dietary toxicity test using northern bobwhite chicks and a commercial formulation of the OP azinphos-methyl. Azinphos-methyl, applied in the field exposure at 770 g/ha, was examined using the EPA quotient method (Matz et al. 1998). Feed contamination was estimated at 40 ppm on insects and there was a presumption of little adverse ecological effects on the bobwhite (Matz et al. 1998). However, survival and brain ChE activity were both significantly lower in comparison to the field control groups (Matz et al. 1998). Further, results of standard laboratory toxicity tests using a dietary concentration of 150 ppm found azinphos-methyl significantly less hazardous than when applied in the field at 770 g/ha (Matz et al. 1998). Further still, the field application of azinphos-methyl at 770 g/ha remained toxic and lethal to bobwhite released onto the field 6 days after spraying, although the calculated half-life for azinphos-methyl in the field was only 2.5 to 4.5 days (Matz et al. 1998). The EPA QM clearly underestimated the risk of mortality to bobwhite chicks in the field (Matz et al. 1998). Matz et al. (1998) concluded that the risks to birds in the field differ from QM

predictions because the QM fails to account for routes of exposure other than oral, environmental variables, and indirect effects, among other factors.

Vyas et al. (2006) conducted subacute dietary toxicity tests with diazinon and Canada goose (*Branta canadensis*) goslings under both field and laboratory conditions. Diazinon's toxicity to goslings was significantly higher in the field than in the lab (Vyas et al. 2006). Laboratory estimates of the dietary diazinon LC_{50} for the technical grade and commercial formulations were 623 and 634 ppm diazinon, respectively (Vyas et al. 2006). The field estimate of the dietary LC_{50} for the commercial formulation was 3.6 ppm diazinon (Vyas et al. 2006). After accounting for differences in moisture content in feed between the lab and field, Vyas et al. (2006) estimated the toxicity of diazinon to goslings was about an order of magnitude higher in the field than in the lab. An influence of the field on the toxicity of diazinon was inferred (Vyas et al. 2006). Vyas et al. (2006) noted that goslings in the field tests were exposed to diazinon via multiple routes and were also subject to environmental stressors that might increase their susceptibility to diazinon.

2.3 Estimation of Contributions to Toxicity from Different Routes of Exposure

2.3.1 Laboratory-based Estimates of Toxicity from Different Routes of Exposure

There has been little investigation of dermal exposure of birds to pesticides, probably because there is no regulatory requirement for such work in avian ecological risk assessment. Some OPs are more toxic via the dermal route relative to the oral route (Schafer, Jr. et al. 1973, Hudson et al. 1979), that the effects of OPs are both delayed and prolonged via the dermal route relative to the oral route (Henderson et al. 1994), and that the physicochemical properties of chemicals influence both their absorption into circulation (Rogers, Jr. et al. 1974) and their

storage in other tissues (Henderson et al. 1994). Schafer, Jr. et al. (1973) compared oral and dermal toxicities of 17 pesticides, primarily OPs, in red-billed weaver birds (*Quelea quelea*) and house sparrows (*Passer domesticus*). Three pesticides, including the OPs dicrotophos and parathion, were of equivalent toxicity via the oral and dermal routes in the weaver bird (Schafer, Jr. et al. 1973). Dicrotophos and fenthion were more toxic to house sparrows via the dermal route than the oral route (Schafer, Jr. et al. 1973). Hudson et al. (1979) compared the toxicity of 18 different OPs via the oral and dermal routes of exposure in mallards. Three OPs, ethoprop, fenitrothion, and methyl parathion, were more toxic dermally than orally in mallards (Hudson et al. 1979). Hudson et al. (1979) recommended a mammalian dermal toxicity index (DTI), describing the relative hazard of dermal to oral exposure, be used as a screening tool to select pesticides for dermal toxicity testing in birds. Henderson et al. (1994) examined the oral and dermal toxicity of three lipophilic OPs in the domestic pigeon (*Columba livia*). The onset of effects from dermal OP exposure was delayed relative to effects resulting from oral exposure (Henderson et al. 1994). Inhibition of plasma ChE from dermal OP exposure was prolonged relative to that observed from oral OP exposure (Henderson et al. 1994). Henderson et al. (1994) inferred the presence of a reservoir within the pigeon body for storage of the dermally absorbed lipophilic OPs and slow release into circulation. Rogers, Jr. et al. (1974) investigated the percutaneous absorption of numerous chemicals through the skin of the foot and into circulation in the red-winged blackbird (*Agelaius phoeniceus*). Absorption into circulation was inversely related to the experimentally determined epidermis:water partition coefficients for the chemicals (Rogers, Jr. et al. 1994). Hydrophilic chemicals entered circulation more readily than lipophilic chemicals (Rogers, Jr. et al. 1974).

Kramer et al. (2002a, 2002b) compared the pharmacokinetics and pharmacodynamics of the intravenous, oral, and dermal routes of exposure of methyl parathion (MP) in rats. In comparing pharmacokinetics, rats were dosed intravenously via injection and orally via gavage with MP at the rate of 1.5 mg/kg, while graded dermal doses of MP in ethanol were applied to clipped, undamaged skin at the rates of 6.25, 12.5, and 25 mg/kg (Kramer et al. 2002a). Comparisons revealed that the pharmacokinetics of MP in rats are complex and dependent upon route of exposure. Peak mean (SE) concentration of MP in rat blood reached 804.9 (52.2) ng/mL only 2.5 minutes following intravenous injection and then declined rapidly to levels not detectable within 36 to 72 hours. The half-life of MP in rats following intravenous injection was 6.6 hours. Peak mean (SE) concentration of MP in rat blood following oral gavage was 31.2 (11.3) ng/mL at approximately 15 minutes following gavage, although the time to peak was highly variable (range 10 to 60 minutes), and then declined in a manner consistent with observation following intravenous injection. The peak concentration of MP in blood following oral gavage was more than an order of magnitude lower than following intravenous injection and oral bioavailability was estimated at only 20%. The pharmacokinetics of MP in rats following dermal exposures differed markedly from exposures to either intravenous or oral doses. Concentration of MP in rat blood following a dermal dose of 6.25 mg/kg increased slowly to a mean (SE) peak of 40.0 (13.0) ng/mL over a period of up to 26 hours and then declined to levels that were not detectable over a period of 74 to 96 hours. At the higher dermal doses of MP of 12.5 and 25 mg/kg, mean (SE) peak concentrations of MP in blood were 110.0 (47.2) and 143.6 (32.6) ng/mL, respectively. The decay of MP concentrations in rat blood at both higher dermal doses was proportionally longer and still detectable at the end of the protocol, 122 hours post-exposure. They inferred limited diffusion across rat skin following dermal exposures that

restricted bioavailability to approximately 50 percent of the applied dose. Whereas the pharmacokinetics of MP in rats following intravenous and oral doses were best described by three-compartment models, the pharmacokinetics of MP in rats following dermal doses were best described by two-compartment models. Dermal exposures altered the pharmacokinetics of MP in rats with respect to the intravenous and oral routes of exposure. Whereas intravenous and oral doses of MP in rats resulted in MP partitioning into a deep lipophilic compartment, MP failed to enter this compartment following dermal exposures. Instead, the primary reservoir for MP in rats following dermal exposures was rat skin itself, from which MP was slowly released. In related work using similar dosing methods, Kramer et al. (2002b) described the pharmacodynamics of MP in rats. The intravenous and oral doses used were 2.5 mg/kg while the graded dermal doses were 6.25, 12, 25, and 50 mg/kg. Cholinesterase activity in rat red blood cells was examined using repeated measures. Peak inhibition of ChE activity following both intravenous and oral exposures occurred rapidly and recovered completely within 30 to 48 hours. In contrast, peak inhibition of rat ChE activity was dose-dependent, developed slowly (>6 hours to peak), and prolonged in duration (>48 hours). At the highest dermal dose, little recovery of ChE activity was apparent until 122 hours post-dose while full recovery of ChE activity took 14 to 21 days.

Qiao et al. (1994) examined the pharmacokinetics of radio-labeled parathion (PA) and its metabolites in weanling pigs following intravenous and dermal doses. Intravenous and dermal doses of PA were equivalent (300 μg) but the intravenous dose was given via an ear vein cannula while the dermal dose was applied in ethanol vehicle over 7.5 cm^2 of clipped, undamaged skin. Parathion was rapidly cleared from blood following the intravenous dose. Greater than 99% of PA was cleared from blood within the first 15 minutes post intravenous dose and neither PA nor its metabolites were detected in blood after 6 hours. The pharmacokinetic model fit to the

observations predicted dynamic, fluctuating concentrations of PA and its metabolites during the first 24 hours post-intravenous dose, then relatively low and stable levels, and complete clearance from pigs within 48 hours. In contrast, PA and its metabolites persisted in pig blood throughout the 168 hour experimental protocol following dermal dosing. The rate limiting pharmacokinetic step was inferred to be partitioning of PA from the vehicle/skin surface into the lipophilic stratum corneum (SC) of the pig skin. They estimated that 90 to 96% of the PA absorbed into the SC would eventually diffuse into the more hydrophilic viable epidermis (VE).

2.3.2 Simulated Field-based Estimates of Toxicity from Different Routes of Exposure

Mineau et al. (1990) compared the responses of zebra finches (*Taeniopygia guttata*) to either graded simulated forest sprays of fenitrothion in a spray chamber or graded oral doses of fenitrothion by gavage. Four different groups of zebra finches (each n=10) were exposed at the fenitrothion spray rates of 38, 51, 139, and 255 g/ha. Five different groups of zebra finches (each n=5) were exposed to fenitrothion oral doses of 0, 5, 10, 20, and 40 mg/kg. Responses of finches to fenitrothion simulated forest spray exposures displayed an obvious threshold effect. Significant reductions in brain ChE activity and body weight were only observed at the highest fenitrothion spray rate. They noted their spray chamber exposures lacked a dietary component and argued instead that an equivalent oral dose rate could be estimated for their spray chamber exposures based upon peak inhibition of ChE activity. The response in the high spray rate group was estimated to be equivalent to the response to an oral gavage dose of approximately 13 mg/kg.

The investigation of Driver et al. (1991) is perhaps the only effort to date to quantify the relative contribution of different routes of exposure to pesticide toxicity in a bird species under

simulated field conditions. Northern bobwhite were exposed to sprays of the OP methyl parathion in an enclosed wind tunnel apparatus containing a cotton crop. Four routes of exposure were isolated in the exposures; inhalation, direct consumption of contaminated feed, indirect consumption from preening, and dermal. Inhalation was isolated by enclosing bobwhite in Gortex® body bags while exposing only the beak. Consumption of contaminated feed was isolated by comparing effects in bobwhite fed dead contaminated mealworms and bobwhite fed dead clean mealworms. Preening was isolated by comparing effects in bobwhite left free to preen with bobwhite wearing neck collars that prevented preening. The dermal contribution was established by measuring effects in free-ranging bobwhite and subtracting the effects found in the other isolated routes of exposure. Methyl parathion was applied to the simulated field at 1.2 kg/ha. Contaminated feed was produced in similar spray applications and then transferred to a clean simulated feed to isolate consumption of contaminated feed. Cholinesterase inhibition was measured in all groups at 1, 4, 8, 24, and 48 hours following application of the OP spray. The contributions to ChE inhibition by the different routes of exposure varied with post spray period. At 1 hour post-spray, the ranking of exposure routes by relative contribution to toxicity was inhalation>preening>dermal and no effects were observed from ingestion of contaminated feed. At 4 hours post-spray, the ranking was preening>ingestion>dermal>inhalation. At each of 8, 24, and 48 hours post-spray, the ranking was dermal>ingestion>preening>inhalation. Ingestion of contaminated feed contributed no more than 20% to total toxicity in the bobwhite in all of the post-spray periods examined. They noted the stark contrast between their findings and the assumption that consumption of contaminated feed is the main route for exposure to pesticides used in avian ERA.

2.3.3 Modeling

There are two principle modeling strategies in use in the study of dermal exposures (Fitzpatrick et al. 2004). First, quantitative structure-activity relationships (QSARs) and quantitative structure-permeability relationships (QSPRs) are used mainly to predict steady-state permeability coefficients for skin in people. QSARs and QSPRs may then be divided into two separate subsets, general and specific. General QSARs/QSPRs have been developed based upon large data sets from a wide variety of compounds without regard for a compound's class or mechanism of action. Specific QSARs/QSPRs are typically focused on either a smaller numbers of compounds within a related class or on the influence of particular physicochemical properties on absorption. The second modeling strategy lies in mathematical modeling of the partitioning and transport processes involved in dermal absorption to predict the extent and rate of absorption through skin.

Potts and Guy (1992) developed one of the earliest general QSAR models of skin permeability. Data on mammalian skin permeability (K_p) from a number of different experimental sources were compiled for use in the model building exercise. The largest and most diverse of the data sources included K_p data for more than 90 compounds of diverse solubility ($-3 \leq \log K_{ow} \leq 6$), varying molecular weight ($18 \leq MW \leq 750$), and different classes and mechanisms of action. Multiple regression was used to identify a relatively simple model for which skin permeability could be predicted (Eq. 2.1):

$$\log K_p = 0.71 \log K_{ow} - 0.0061 MW - 6.3 \qquad \text{(Eq. 2.1).}$$

This model fit the largest and most diverse of the data sets fairly well ($r^2=0.67$). The model also offered some mechanistic insight. Permeation through mammalian skin was consistent with permeation through a single biological membrane except that permeation through mammalian

skin was much slower than through a single membrane as the diffusion pathlength through mammalian skin was much longer than that through a single membrane.

In contrast, Moss and Cronin (2002) developed a specific QSRP upon re-analyzing published steroid K_p data on percutaneous absorption. They noted that numerous steroids had appeared as outliers in earlier QSPR models and that the quality of the steroid data used in developing these models had been questioned. They developed a specific QSPR model for the permeability of skin to steroids based upon new steroid K_p data. This new QSPR model for steroids (Eq. 2.2)

$$\log K_p = 0.74 \log K_{ow} - 0.0091MW - 2.39 \qquad \text{(Eq. 2.2)}$$

was both highly significant and consistent with prior published models. They concluded that steroids permeate through skin in the same manner as the majority of exogenous compounds studied.

QSAR/QSPR methods have not been limited to predictions of mammalian K_p values under steady state condition. Cleek and Bunge (1993), for example, developed a generalized method that allowed for the inclusion of dermal absorption under non-steady state conditions when the compound is first being absorbed into the stratum corneum and before it has entered circulation. However, a comprehensive discussion of QSARs/QSPRs is beyond the scope of this exercise. The review of Moss et al. (2002) will serve in this capacity as it describes many of the QSARs/QSPRs developed to model skin permeation. Further, it is at least as important to understand the strengths and weaknesses of the different approaches in QSAR/QSPR modeling as it is to be familiar with all of the models developed. Geinoz et al. (2004) found that many QSARs/QSPRs had been developed using data compiled from different sources and that among-source heterogeneity in experimental conditions contributed unexplained variance in the models.

They further noted that specific QSAR/QSPR models that are confined to compounds within classes are more reliable than more general models but also more limited in applicability (Geinoz et al. 2004). Lian et al. (2008) compared what they described as "empirical" and "mechanistic" QSARs/QSPRs. They noted that the more complex models often contained terms describing molecular structure which are only empirically related to skin permeation and reveal little mechanistic information. They found that the best predictions resulted from the mechanistic model of Mitragotri (2002) and that the second best predictions resulted from the empirical model of Potts and Guy (1992). The mechanistic model of Mitragotri (2002) was based in scaled particle theory for solute diffusion in lipid bilayers and used log K_{ow} and solute radius to predict skin permeability. Lian et al. (2008) noted that, although Potts and Guy (1992) and Mitragotri (2002) took different approaches in developing their models (empirical and mechanistic, respectively), they shared two features. Both models assume that the lipid matrix within skin is the permeation pathway involved in dermal absorption and both models rely on measures of log K_{ow} and solute size in making their predictions (Lian et al. 2008). Lian et al. (2008) concluded that additional complexity developed empirically did not result in more accurate predictions of skin permeation.

Others have used QSARs/QSPRs to examine how absorption varies with log K_{ow} (Yano et al. 1986, Hinz et al. 1991, Zhang et al. 2009). Yano et al. (1986) measured the absorption of two sets of homologous compounds, 8 salicylates and 10 non-steroidal anti-inflammatory drugs (NSAIDs), in human subjects. Two square centimeters of forearm skin was demarcated on healthy male human subjects. The test compounds were then applied to demarcated skin in volatile solvents using microsyringes at a uniform dose of 0.5 mg for each compound. Demarcated skin was covered with an aluminum foil patch for 4 hours after which the

unabsorbed compound was recovered from both the patch and demarcated skin with ether. The percent absorbed (%Abs) for each compound was estimated from the recoveries determined following removal of patches. Quadratic polynomial equations were fit to the scatter plots of log K_{ow} versus log %Abs for the salicylates and NSAIDs data sets (Eq. 2.3 and 2.4, respectively),

$$\log \%Abs = -0.323(\log K_{ow})^2 + 1.575(\log K_{ow}) + 0.010 \quad (r^2=0.931, \text{Eq. 2.3})$$

$$\log \%Abs = -0.203(\log K_{ow})^2 + 0.985(\log K_{ow}) + 0.514 \quad (r^2=0.929, \text{Eq. 2.4}).$$

The optimal log K_{ow} for dermal absorption was approximately 2.4 for both the salicylates and NSAIDs.

Hinz et al. (1991) measured the skin permeation rate of radio-labeled phenol and 10 radio-labeled *para*-substituted phenols across full-thickness isolated hairless mouse skin in vertical, glass flow-through diffusion cells. Log K_{ow} for the phenols varied by more than 3 orders of magnitude. Skin was excised and used immediately following euthanasia of the mice. All phenols were applied to 3.1 cm^2 of skin in an acetone vehicle at the rate of 4 $\mu g/cm^2$. The receptor chamber contained phosphate-buffered saline (PBS) having an hourly turnover rate. Receptor solution was sampled every hour or two over a 48 hour period. Skin permeation for the phenols was characterized by the maximum skin permeation rate, J_{max}. Hinz et al. (1991) fit both parabolic and bilinear models to the scatter plot of log K_{ow} versus log J_{max}. Statistical analysis indicated that both models adequately described variation in the data. The fit of the parabolic model (Eq. 2.5),

$$\log J_{max} = -0.30(\log K_{ow})^2 + 1.35(\log K_{ow}) - 0.14 \quad (r^2=0.92, \text{Eq. 2.5}),$$

to the data was highly significant ($p<0.001$) and the model was consistent with a change in the rate-limiting absorption process from diffusion through the SC to partitioning across the SC/VE boundary.

Similarly, Zhang et al. (2009) fit parabolic and bilinear equations to observations of J_{max} for a set of phenols through full-thickness human abdominal skin. Log K_{ow} for the phenols ranged from 1.95 to 3.52. Human skin was obtained from a single individual and mounted in horizontal, glass static diffusion cells. Dosing solutions in the donor chambers were described as providing an "infinite" dose as they were saturated phenol solutions of PBS in very large volumes relative to the skin diffusion area. Samples of the PBS in the receptor chamber were collected and replaced with fresh PBS at regular intervals over the next 7 hours. J_{max} was equated to the steady-state skin permeation rate observed for each phenol. Both parabolic and bilinear models were fit to the scatter plot of log J_{max} versus log K_{ow} for the phenols under study. Here the fit of the bilinear model (r^2=0.86) was somewhat better than the fit of the parabolic model (Eq. 2.6)

$$\log J_{max} = -1.2(\log K_{ow})^2 + 6.5(\log K_{ow}) - 7.2 \quad (r^2=0.74, \text{Eq. 2.6}).$$

They noted that their findings were largely consistent with those of Hinz et al. (1991) described previously. However, they differed from Hinz et al. (1991) in the inference made regarding the processes responsible for the convex shape of the model. They had deliberately selected phenols that differed little in MW or hydrogen bonding ability and concluded that the solubility of the phenols in the SC alone explained the shape of the relationship.

Mathematical models of diffusion and partitioning processes, the second approach to modeling dermal absorption, also vary in their complexity from the simple, general pharmacokinetic (PK) model of percutaneous absorption of Guy et al. (1982) to the much more complex but specific physiologically-based pharmacokinetic pharmacodynamic (PBPKPD) model of chlorpyrifos exposure and effects of Timchalk et al. (2002). The model of Guy et al. (1982) is a 4-compartment general model for predicting the percutaneous absorption of a variety

of compounds. It describes the excretion of topically applied testosterone, benzoic acid, and hydrocortisone over time as being a function of 4 first-order rate constants, k_1 through k_4 (Guy et al. 1982). The rate constants k_1, k_2, k_3, and k_4 represent the rate of compound diffusion across the SC, the rate of compound diffusion across the VE, the delay rate of partitioning across the SC-VE boundary associated with the SC's reservoir effect (i.e. reflecting the extent to which a compound has a greater affinity for the lipophilic SC over the hydrophilic VE), and the elimination rate of the compound from dermal capillaries into urine, respectively (Guy et al. 1982). Guy et al. (1982) noted that the ratio of k_3/k_2 for the three compounds under study were consistent with their relative hydrophobicities. This model was later validated for 11 additional compounds of widely varying solubility properties leading to the conclusion that the ratio k_3/k_2 agreed reasonably well with octanol:water partition coefficients for the compounds under study (Guy et al. 1985). In contrast, the model of Timchalk et al. (2002) possesses 20 compartments, 8 rate constants, and parameters for both blood flow and contaminant concentration in various tissues in modeling the multiroute pharmacokinetics, pharmacodynamics, metabolism, and excretion of chlorpyrifos in rats and man. Timchalk et al. (2002) concluded their model was capable of estimating tissue-specific chlorpyrifos dose and ChE inhibition within a variety of exposure scenarios.

Mathematical models of dermal exposure have apparently never been developed for birds. However, there are avian PK models of alimentary exposure to pharmaceuticals (Pollet et al. 1985, Cortright et al. 2009) and persistent organic pollutants (Clark et al. 1987, MacLachlan 2010). The PBPK models of Pollet et al. (1985) and Cortright et al. (2009) were developed for midazolam and chlortetracycline, respectively, in addressing concerns regarding the veterinary use of pharmaceuticals. The simple PK model of Clark et al. (1987) was developed to simulate

the kinetics of organochlorine (OC) residues in herring gulls (*Larus argentatus*) to better understand and predict temporal trends in OC concentrations in lakes where herring gulls feed. The PBPK model of MacLachlan (2010) was developed to predict the accumulation of lipophilic pesticides in poultry. The author knows of no similar mathematical model of the exposure of birds to any compound via the dermal route. The mammalian literature is therefore the source of mathematical models of dermal absorption and the following select articles are relevant to this endeavour.

A PBPK model for the *in vitro* absorption of parathion, methyl parathion, and fenthion through pig skin isolated within a flow-through diffusion cell was developed by van der Merwe (2006). The model was mechanistic and rich in detail, although limited in application to the experimental conditions, and specific to OP pesticides listed. Among the phenomena studied were the effects of solvent and removal of the SC on absorption. The authors noted apparent deviations from the first-order kinetics predicted by Fick's law in their observations with parathion and fenthion. Diffusivity and mass transfer were altered depending upon the solvent and solute concentrations used. They further commented that solvent-solute interactions that can be anticipated with absorption across biological barriers were incompatible with the mechanism of Brownian motion that forms the basis for Fick's law. More importantly, they noted that for these 3 small, lipophilic OPs, absorption rate was limited by the diffusion of the OPs through skin as opposed to partitioning of the OPs into skin from the solvent. Only a small fraction of the OPs that partitioned into skin were absorbed into the receptor solution during their 8 hour experimental periods. They stated that small, lipophilic compounds such as these OPs are likely to be stored in a skin reservoir following topical application and only slowly absorbed into circulation.

Mosquin et al. (2009) discussed the benefits of multiple biomarkers and multiple sampling periods in using PBPK models to infer exposures from biomarker data. This ‘inverse approach’ stands in contrast to the typical use of PBPK models and biomarker data to infer tissue concentrations of a compound given a known exposure. They employed a modified version the PBPKPD model of Timchalk et al. (2002) for chlorpyrifos in rats and humans and a simulated biomarker data set for two contrasting exposure scenarios. The two exposure scenarios simulated were one-time acute exposures to chlorpyrifos via either the oral or dermal route. Although the complete simulated biomarker data set included measures of 5 different biomarkers at 4 different time periods following exposure, only portions of the data set were used with the model to assess how the addition of more biomarkers or more sampling times improved model utility in reconstructing exposures. They found that both additional biomarkers and additional sampling times added information to the analysis but that the extra sampling times were more important to reconstructing exposures.

Chapter 3

3.0 SIMULATED FIELD EXPOSURES OF COTURNIX QUAIL TO ORGANOPHOSPHATE INSECTICIDE SPRAYS I: CONTRIBUTIONS TO TOXICITY FROM VARIOUS ROUTES OF EXPOSURE

3.1 INTRODUCTION

The current screening level ecological risk assessment quotient method used to evaluate the hazard to wild birds from the use of insecticidal sprays, including the anticholinesterase organophosphorus (OP) and carbamate (CM) compounds, relies on an exposure model that assumes consumption of contaminated food is the only route of exposure (Tiebout and Brugger 1995). There are a number of lines of evidence that suggest dermal and other routes of exposure occur in the field and pose hazards to wild birds with the use of insecticidal sprays. This evidence includes comparisons of standard toxicity tests under field and laboratory conditions (Tiebout and Brugger 1995, Matz et al. 1998, Vyas et al. 2006), avian dermal toxicity studies (Schafer, Jr. et al. 1973, Rogers et al. 1974, Hudson et al. 1979, Henderson et al. 1994), the use of non-dietary routes of exposure in the design of avicides (Schwab 1968, Pope and Ward 1972, Schafer et al. 1984, Johns et al. 1992, Shumake and Scott 1996), the presence of insecticide residues on and in avian skin following insecticide spray application (Vyas et al. 2004, Vyas et al. 2007), studies of the field record of avian mortality from insecticidal sprays (Mineau 2002), and controlled exposures to insecticidal spray in chambers (Mineau et al. 1990) and under simulated field conditions (Driver et al. 1991).

Tiebout and Brugger (1995) noted several assumptions of the quotient method, including that consumption of contaminated feed is the only route of exposure. They noted that the quotient method, while simple to apply and interpret, can also be insensitive, imprecise, and inaccurate. Comparisons of field and laboratory toxicity tests using OP pesticides and birds

found that the quotient method underestimated the risks to birds exposed to OPs in the field (Matz et al. 1998, Vyas et al. 2006). Matz et al. (1998) compared the results of a controlled field study with a standard laboratory dietary toxicity test using northern bobwhite chicks and a commercial formulation of the OP azinphos-methyl. Azinphos-methyl, applied in the field exposure at 770 g/ha, was examined using the quotient method. Feed contamination was estimated at 40 ppm on insects and there was a presumption of little adverse ecological effects on the bobwhite. However, survival and brain ChE activity were both significantly lower in comparison to the field control groups. Further, results of standard laboratory toxicity tests, using a dietary concentration of 150 ppm, found azinphos-methyl to be significantly less hazardous than when applied in the field at 770 g/ha. The field application of azinphos-methyl at 770 g/ha remained toxic and lethal to bobwhite released onto the field 6 days after spraying, although the calculated half-life for azinphos-methyl in the field was only 2.5 to 4.5 days. The quotient method clearly underestimated the risk of mortality to bobwhite chicks in the field. They concluded that the risks to birds in the field differ from quotient method predictions because the method fails to account for routes of exposure other than oral, environmental variables, and indirect effects, among other factors.

Vyas et al. (2006) conducted subacute dietary toxicity tests with diazinon and Canada goose goslings under both field and laboratory conditions. Diazinon's toxicity to goslings was significantly higher in the field than in the lab. Laboratory estimates of the dietary diazinon LC_{50} for technical grade and commercial formulations were 623 and 634 ppm diazinon, respectively. The field estimate of the dietary LC_{50} for the commercial formulation was 3.6 ppm diazinon. After accounting for differences in moisture content in feed between the lab and field, they estimated the toxicity of diazinon to goslings was about an order of magnitude higher in the field

than in the lab. An influence of the field on the toxicity of diazinon was inferred. They noted that goslings in the field tests were exposed to diazinon via multiple routes and were also subject to environmental stressors that might increase their susceptibility to diazinon.

Avian dermal exposure studies have revealed that some OPs are more toxic via the dermal route than by the oral route (Schafer Jr. et al. 1973, Hudson et al. 1979), that the effects of OPs are both delayed and prolonged via the dermal route relative to the oral route (Henderson et al. 1994), and that the physicochemical properties of chemicals influence both their absorption into circulation (Rogers, Jr. et al. 1974) and their storage in other tissues (Henderson et al. 1994). Schafer, Jr. et al. (1973) compared the oral and dermal toxicities of seventeen pesticides, primarily OPs, in red-billed weaver bird and house sparrow. Three pesticides, including the OPs dicrotophos and parathion, were of equivalent toxicity via the oral and dermal routes in the weaver bird. Dicrotophos and fenthion were more toxic to house sparrows via the dermal route than the oral route. Hudson et al. (1979) compared the toxicity of 18 different OPs via the oral and dermal routes of exposure in mallards. Three OPs, ethoprop, fenitrothion, and methyl parathion, were found more toxic dermally than orally in mallards. They recommended a mammalian dermal toxicity index (DTI) describing the relative hazard of dermal to oral exposure be used as a screening tool to select pesticides for dermal toxicity testing in birds. Henderson et al. (1994) examined the oral and dermal toxicity of three lipophilic OPs in the domestic pigeon. The onset of effects from dermal OP exposure was delayed relative to effects resulting from oral exposure. Inhibition of plasma ChE from dermal OP exposure was prolonged relative to that observed from oral OP exposure. They inferred that a reservoir existed within the pigeon body for storage of the dermally absorbed lipophilic OPs and slow release into circulation. Rogers, Jr. et al. (1974) investigated percutaneous absorption of numerous chemicals through the skin of the

foot and into circulation in the red-winged blackbird. Absorption into circulation was inversely related to the experimentally determined epidermis:water partition coefficients for the chemicals. Hydrophilic chemicals entered circulation more readily than lipophilic chemicals.

Avicides are used in the control of bird pests. Many routes of exposure are employed in the development and use of avicides (Schwab 1968, Pope and Ward 1972, Schafer, Jr. 1984, Johns et al. 1992, Shumake and Savarie 1996). Early efforts to control starlings and blackbirds in California used 3-chloro *p*-toluidine hydrochloride, an organochlorine, as a contact avicide in roost sprays (Schwab 1968). Schwab (1968) inferred several routes of exposure as contributing to the lethality of the organochlorine studied. The OPs fenthion and parathion have been used widely in Africa as contact avicides in roost sprays in the control of red-billed quelea (Pope and Ward 1972). Pope and Ward (1972) found that a commercial formulation of fenthion applied to feathers of the back of red-billed quelea resulted in LD_{50}s of 80 to 220 mg/kg depending upon dilution and droplet size. The droplet size and solvent used to deliver fenthion to the bird were important as they influenced the ability of fenthion to wick through the plumage to the skin. Fenthion is also used as an avicide in wicked perches (Schafer, Jr. 1984). Dermal exposure is utilized as the perches deliver fenthion to the feet of target bird species. The use of 3-chloro-4-methylbenzenamine, an organochlorine, as a respirable aerosol spray avicide against starlings and blackbirds was investigated by Johns et al. (1992). They found that ocular and body exposures were larger contributors to lethality of the organochlorine than inhalation. Shumake and Savarie (1996) explored use of OP-contaminated nest material in the control of weaver finches. A commercial insecticide formulation of fensulfothion was found to be the most effective avicide in nest material. Male weaver finches were disproportionately targeted due to their role in nest building.

The presence of residues on, or in, avian skin has rarely been examined. OP pesticide sprays are readily transferred from vegetation to bird feet and feathers (Vyas et al. 2004, Vyas et al. 2007). Vyas et al. (2004) exposed Canada goose goslings to diazinon-sprayed turf by housing them on experimental turf plots, upon which they walked and grazed. All diazinon-exposed goslings died within 24 hours of exposure and diazinon residues were detected on their feet even after 7 days of decomposition. They proposed that feet be used for residue analyses for the diagnosis of OP poisoning when other tissues are unavailable. Vyas et al. (2007) released brown-headed cowbirds (*Molothrus ater*) into an aviary containing azinphos-methyl-sprayed apple trees. Cowbirds released into the aviary at 1 hour post-spray were sampled at 36 hours and 7 days post-spray while cowbirds released into the aviary at 4 days post-spray were sampled only at 7 days post-spray. The majority of cowbirds sampled from each exposure group had detectable residues on their skin plus feathers and feet. They claimed their finding of residues on these tissues demonstrated the importance of dermal exposure in avian ecological risk assessment.

The field record of avian mortality suggests that exposure routes other than oral contribute to bird kills (Mineau 2002). Mineau (2002) developed logistic regression models to predict the probability of bird kills from spray application of anticholinesterase pesticides based upon a review of field studies following pesticide application. Three models were developed, one for each of field crops or pastures, forests, and orchards. Three independent variables, the toxic potential (TP) as a measure of acute oral toxicity and spray rate, an avian DTI, and Henry's law constant (HLC) as a surrogate for potential inhalation exposure, all improved the models' predictive ability. Generally, the ranking of the independent variables from most significant to least significant in their ability to improve model predictions was TP>DTI>HLC. Mineau (2002) stated that for two anticholinesterase pesticides of equal oral toxicity and spray rate the one

having the higher DTI was more likely to cause bird mortality and concluded that the models demonstrate the need to incorporate dermal, and in some instances inhalation toxicity, into avian ecological risk assessment.

The field exposure of a small songbird to the spray of an OP insecticide was simulated under controlled conditions by Mineau et al. (1990) using zebra finches, fenitrothion and an experimental spray chamber. A spray exposure to fenitrothion at 255 g/ha was equated to an oral dose of 13.3 mg/kg based upon matching acetylcholinesterase inhibition in the brains of spray- and oral-exposed zebra finches. However, exposures at lower spray rates did not produce the significant inhibition of brain acetylcholinesterase that had been anticipated (Mineau et al. 1990). They noted that their laboratory observations at these lower spray rates were in contrast to observations from the field and suggested that multiple routes of exposure may contribute to toxicity at low field application rates.

Driver et al. (1991) attempted to quantify the relative contribution of different routes of exposure to pesticide toxicity in a bird species under simulated field conditions. Northern bobwhite were exposed to sprays of the OP methyl parathion in an enclosed wind tunnel apparatus containing a cotton crop. Four routes of exposure were isolated in the exposures; inhalation, direct consumption of contaminated feed, indirect consumption from preening, and dermal. Inhalation was isolated by enclosing bobwhite in Gortex® body bags while exposing only the beak. Consumption of contaminated feed was isolated by comparing effects in bobwhite fed dead contaminated mealworms and bobwhite fed dead clean mealworms. Preening was isolated by comparing effects in bobwhite left free to preen with bobwhite wearing neck collars that prevented preening. The dermal contribution was established by measuring effects in free-ranging bobwhite and subtracting the effects found in the other isolated routes of exposure.

Methyl parathion was applied to the simulated field at 1.2 kg/ha. Contaminated feed was produced in similar spray applications and then transferred to a clean simulated feed to isolate consumption of contaminated feed. Cholinesterase inhibition was measured in all groups at 1, 4, 8, 24, and 48 hours following application of the OP spray. The contributions to ChE inhibition by the different routes of exposure varied with post spray period. At 1 hour post-spray, the ranking of exposure routes by relative contribution to toxicity was inhalation>preening>dermal and no effects were observed from ingestion of contaminated feed. At 4 hours post-spray, the ranking was preening>ingestion>dermal>inhalation. Thereafter, at each of 8, 24, and 48 hours post-spray, the ranking was of the contribution from different exposure routes was dermal>ingestion>preening>inhalation. Ingestion of contaminated feed contributed no more than 20% to total toxicity in the bobwhite in any of the post-spray periods examined. Driver et al. (1991) noted the contrast between their findings and the assumption of avian ecological risk assessment that consumption of contaminated feed is the only route for exposure to pesticides.

The body of knowledge on dermal exposures of birds to pesticides and other compounds appears modest in comparison to that developed in studies of dermal exposures with mammals. The dermal route of exposure is particularly important in human health risk assessment of occupational exposures to pesticides and in the design of pharmaceuticals (Fitzpatrick et al. 2004). Generally, compounds with balanced lipid-water solubility are more readily absorbed through skin than compounds that are either extremely hydrophilic or extremely lipophilic (Lane et al. 2012). This phenomenon is observed because skin consists of a series of hydrophobic and hydrophilic barriers, the stratum corneum (SC) and viable epidermis (VE), respectively, between the external environment and the circulatory system (Lane et al. 2012). Extremely hydrophilic compounds do not readily partition into the SC while extremely lipophilic compounds enter the

SC with relative ease but then do not readily partition into the VE (Lane et al. 2012). In the latter case, a lipophilic compound will accumulate in the SC (Lane et al. 2012). This reservoir effect for lipophilic compounds in the SC is well known (Miselnicky et al. 1988), having been demonstrated with steroids as early as 1963 (Vickers 1963). Further, as the lipophilicity of a compound increases, so do the reservoir effect (Miselnicky et al. 1988) and retention time in the SC (Yagi et al. 1998). Given the mechanisms involved in dermal absorption, it is effectively impossible for a bolus-type dose to be absorbed through skin (Guy 1996). Instead, a dermal dose results in an absorption profile that is relatively steady and prolonged in comparison to the peaked profile of short duration associated with equivalent oral bolus-type doses (Subedi et al. 2010). This relationship is apparent in the observations of Kramer et al. (2002b) who compared cholinesterase activity in rats following either oral or dermal doses of the OP methyl parathion (MP). They found that the toxicodynamics of MP differed greatly between the two routes of exposure. Oral doses of MP (2.5 mg/kg) in rats produced maximum inhibition of ChE activity typically within an hour and activity recovered to normal levels typically within 30 to 48 hours. In comparison, dermal doses (6.25, 12, 25, and 50 mg/kg in ethanol) applied to skin (2 to 2.5 cm^2) on the necks of rats from which hair had been carefully clipped resulted in dose-dependent inhibition of ChE activity of a magnitude comparable to the oral dose. However, peak inhibition developed after a delay of 12 to 26 hours and complete recovery of ChE activity was delayed beyond 122 hours. They inferred that a reservoir exists within rat skin and gradual release of MP from skin into circulation as being responsible for the delayed and prolonged inhibition of ChE activity in rats following their dermal exposures. These effects of dermally absorbed MP in rats (Kramer et al. 2002) are consistent with the previously described observations of dermally absorbed lipophilic OPs in pigeons (Henderson et al. 1994).

This work tests the assumption that consumption of contaminated feed is the only route of exposure of wild birds to insecticidal sprays in a set of replicated, controlled spray exposure experiments conducted on a simulated field of barley using commercial formulations of four different OP compounds of varying physicochemical properties. Further, this work seeks to identify what routes of exposure, if not consumption of contaminated feed, actually do contribute to the toxicity of OP sprays under field conditions.

3.2 MATERIALS AND METHODS

3.2.1 Experiment

3.2.1.1 Experimental Species

Coturnix quail (*Coturnix coturnix*) were purchased from Fircrest Farms Ltd. (Langley, BC, Canada). All quail used in the study were males between 6 and 26 weeks of age. Each quail was assigned a number and tagged for identification purposes using a commercial poultry tagger shortly after their arrival.

3.2.1.2 Animal Care and Housing

The study was conducted in accordance with Protocol #20070071 approved by the Research Ethics Board of the University Committee on Animal Care and Supply at the University of Saskatchewan. All quail were housed indoors at the Animal Care Unit in the Western College of Veterinary Medicine, University of Saskatchewan. Quail were provided a commercial, medicated (amprolium 125 mg/kg), crumbled turkey starter feed (Federated Co-operatives Limited, Saskatoon, SK, Canada) and water *ad libidum*. A 8 hours light:16 hours dark cycle was maintained throughout the study.

3.2.1.3 Experimental Designs

A factorial experimental design was used to investigate the exposure to and toxicity of different OP insecticidal sprays to quail under varying simulated field exposure scenarios (Figure 3.1). The design was grouped by four factors; OP insecticide (4 levels), exposure scenario (5 levels), time (2 levels), and replicate (3 levels). The sample size was five quail per group and the study required six hundred birds. Quail were randomly assigned to different treatment groups. The dependent variables measured were cholinesterase activity in brain and plasma. Cholinesterase activity in brain was measured once in each bird at the end of the protocol. Cholinesterase activity in plasma was measured twice for each bird, once before exposure and once at the end of the protocol.

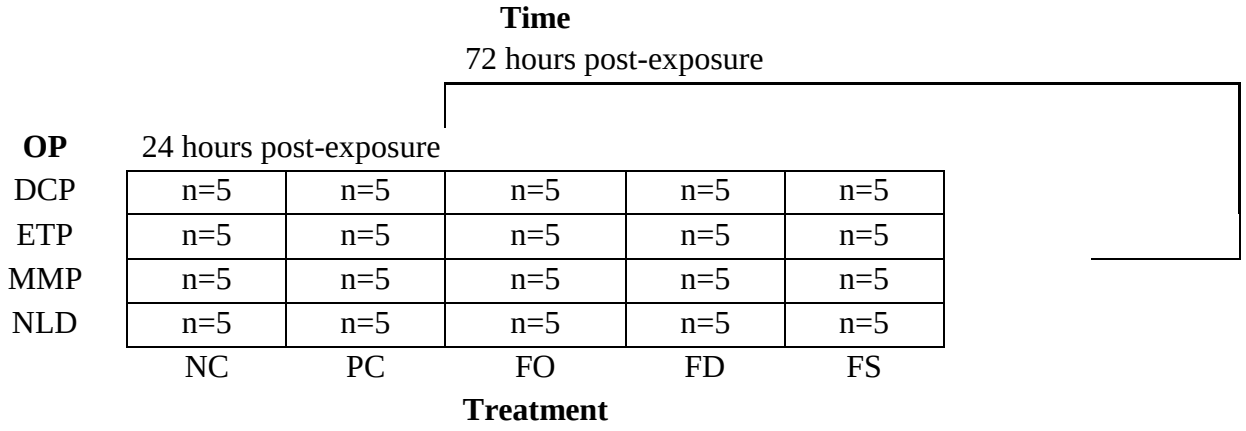


Figure 3.1. Experimental factorial design for a single replicate of the simulated field investigation illustrating grouping of quail by different levels of the factors Time (24 and 72 hours post-exposure), Treatment (negative control (NC), positive control (oral gavage) (PC), field only (FO), field plus diet (FD), and field plus spray (FS)), and Organophosphate (OP) insecticide (dicrotophos (DCP), ethoprop (ETP), methamidophos (MMP), and naled (NLD)). The fourth factor, Replicate, for which there are three levels, is not illustrated.

3.2.1.4 Organophosphate Insecticides

Organophosphate (OP) insecticides were selected representing a range of solubility properties from hydrophilic to lipophilic. Commercial spray formulations of dicrotophos (DCP; 2-dimethyl cis-2-dimethylcarbamoyl-1-methylvinylphosphate)(BIDRIN®, 82.0% DCP by weight, Amvac Chemical Corporation), ethoprop (ETP; O-ethyl S,S-dipropyl phosphorodithioate)(MOCAP®, 69.6% ETP by weight, Bayer CropScience), methamidophos (MMP; O,S-dimethylphosphora-midothiolate)(MONITOR®, 480 g MMP/L, Bayer CropScience), and naled (NLD; 1,2-dibromo-2,2-dichloroethyl dimethyl phosphate)(DIBROM®, 864 g NLD/L, United Agri Products Canada Inc.) were used in the experiment. All formulations were either suspension or emulsifiable concentrates. Solubility and other physicochemical properties varied among the OP insecticides used (Table 3.1).

Table 3.1. Estimated physicochemical and toxicological properties of dicrotophos (DCP), ethoprop (ETP), methamidophos (MMP), and naled (NLD).

Compound	DCP	ETP	MMP	NLD
log K_{ow}[1]	–0.49	3.59	–0.80	1.38
MW[2]	237.2	242.3	141.1	380.8
LD_{50}[3] (mg/kg)	5.7	7.5	15	50

[1]octanol:water partition coefficient
[2]molecular weight
[3]estimated median lethal oral dose in Japanese quail, except for NLD where the estimated median lethal dose is in Northern bobwhite

3.2.1.5 Simulated Field Set Up and Spray Application

A 1.8 meter by 5.4 meter ‘table-top’ simulated field of barley was constructed for use in spray exposures (Appendix A). Rows of holes were cut into the ‘table-top’ to receive barley seedlings and simulate crop rows. Barley seedlings were grown to about 3 weeks age and 20 to 25 centimeters height in forestry seedling cones (Ray Leach “Cone-tainer”™ Single Cell

System, Tangent, OR, US) under greenhouse conditions, then transferred to the spray room as needed. Locally available organic soil was spread over the simulated field around the barley seedlings to a depth of 3 to 4 cm. A set of cage panels was constructed to divide the field into three pens, each 1.8 meters by 1.8 meters, following application of pesticide spray for concurrent exposures in three scenarios; 1) field only (FO), 2) field plus dietary (FD), and 3) field plus spray (FS), each described in later subsections.

The simulated field spray exposures were completed in the experimental spray room at Agriculture and Agri-Food Canada (AAFC) on the University of Saskatchewan campus. All surfaces around the simulated field in the spray room were covered with plastic drop sheets prior to the spray application. Spray was applied to field using five flat fan nozzles (XR Teejet 8006VS) on a spray boom attached to a ceiling-mounted track to simulated vehicular spray application. The spray mix was applied to the simulated field at the rate of 100 litres per hectare. Generally, the cage was erected and quail placed in pens within 30 minutes following spray application.

Application rates for use in the spray exposures were 2550 g DCP/ha, 1310 g ETP/ha, 7800 g MMP/ha, and 33000 g NLD/ha. Application rates were estimated to produce approximately 60% inhibition of plasma cholinesterase at 24 hours post-exposure based upon dose-response relationships established in pilot studies.

Three replicate sprays were completed with each pesticide. The field was disassembled and all contaminated soil, vegetation, and plastic sheets removed from the spray room following each exposure. The disassembled field, cage panels, and spray room were washed thoroughly with a high pressure spray washer between each spray.

3.2.1.6 Fasting

Quail in all exposure groups, including both field exposure groups and control groups, were deprived of feed for a period of 24 hours prior to exposure.

3.2.1.7 Field Only (FO) Exposure

Quail were placed within a single pen for 6 hours in the FO exposure. Exposure was to sprayed soil and barley. Exposures in all replicates occurred within the time window of 0930 hours and 1630 hours, with only slight variation among replicates. Ten quail were exposed in each replicate spray. Following exposure, quail were returned to housing where they were held in isolation pending tissue sampling and sacrifice.

3.2.1.8 Field plus Dietary (FD) Exposure

The commercial feed used in housing was added to a pen at the rate of 30 g/sq. m prior to the spray being applied in the FD exposures. Exposure was to sprayed soil, barley, and feed. These exposures were otherwise consistent with FO exposures.

3.2.1.9 Field plus Spray (FS) Exposure

Quail in the FS exposure were contained on the simulated field in open mesh cages during application of the spray and then introduced onto the field within a pen. No feed was added to the pen. Exposure was to direct spray, then sprayed soil and barley. These exposures were otherwise consistent with FO exposures.

3.2.1.10 Gut Content (GC) Spray Exposure

A separate set of spray exposures were conducted on the simulated field to investigate feed consumption associated with each of the insecticides. A reduced scale simulated field was erected to provide a single pen. The pen was then subdivided into two 1.8 x 0.9 m subpens. Each subpen was used in either a FO or FD exposure (each n=5) that was otherwise consistent with experimental protocols. Quail were euthanized promptly following the 6 hour exposure and the contents of crops, gizzards, and small intestine collected for determination of the wet mass of gut contents in each quail. These exposures were not replicated save for a pilot GC spray exposure using ETP at 2100 g/ha.

3.2.1.11 Control Groups

Both negative control (NC) and positive control (PC) groups were included in each replicate. Negative control quail remained in housing as a pilot study using a water spray failed to find an effect of the simulated field exposure on cholinesterase activity in either of brain or plasma. Positive control quail were exposed by oral gavage at the Toxicology Centre on the day of spray exposure between 1300 and 1400 hours, then returned to housing and held in isolation. Dose rates for DCP (1.9 mg/kg), ETP (2.5 mg/kg), and MMP (5.0 mg/kg) were at one-third the estimated LD_{50} for each compound (Environment Canada, unpublished data) provided by a coauthor (Mineau). The dose rate for NLD (12 mg/kg) was reduced from the initial estimate (17 mg/kg) as it caused 60% mortality in quail of a pilot oral dose study.

3.2.2 Exposure and Toxicity

3.2.2.1 Tissue Collection and Storage

Blood samples were obtained from each quail prior to initiating the experiment and upon termination of the experiment. Approximately 400 µL of blood was collected by jugular veinipuncture using a heparinized 1 mL syringe with 27 gauge needle at each time. Blood samples were centrifuged immediately at 2400xG for 10 minutes at 4°C to separate plasma from red blood cells. Plasma was collected and stored at -80°C until prepared for assay. Quail were euthanized by cervical dislocation following collection of the terminal blood sample. Brain was dissected from each carcass and stored at -80°C until prepared for assay.

3.2.2.2 Sample Preparation

Plasma samples were diluted (dilution factor (DF)=12) in 0.5 M phosphate buffer (pH 8) prior to assay. Brain samples were first homogenized in 0.5 M phosphate buffer containing 0.5% Triton X-100 (v/v), while in an ice bath, using a Sonic Dismembrator Model 100 tissue homogenizer (Fisher Scientific) for 40 seconds, then further diluted (total DF=168) in the same buffer prior to assay.

3.2.2.3 Cholinesterase Assay

Determination of total ChE activity in tissues was accomplished using the assay of Ellman et al. (1961) as modified for a microplate reader by Gard and Hooper (1993). Briefly, enzyme activity was measured colorimetrically at 412 nm using acetylthiocholine iodide (AThCh)(>99%, Sigma-Aldrich, UK) as a substrate. Buffer (0.5 M phosphate, pH=8), a solution of 5,5-dithiobis(2-nitrobenzoic acid) (DTNB)(Sigma-Aldrich, USA) in buffer (0.5 M phosphate,

pH=7) (FC=$3.23x10^{-4}$ M), sample preparation, and substrate in buffer (0.5 M phosphate, pH 8) (FC=$1x10^{-3}$ M) were added to 96 well microtiter plates (Nalge Nunc International, Rochester, NY, USA)(total volume=260 µL). Buffer was maintained at 25°C while reagent solutions and samples were maintained in ice baths. Substrate was added last, immediately prior to assay, to initiate the reaction. ChE assay results were read on a SpectraMax® 190 microplate reader (Molecular Devices Corporation, Sunnyvale, CA, USA) set in absorbance mode and at 25°C**.** The contribution of BChE to total ChE activity in quail plasma was determined in a subset of the experimental birds using the selective BChE inhibitor tetraisopropyl pyrophosphoramide (iso-OMPA)(Sigma-Aldrich, USA). Samples were incubated with iso-OMPA (incubation concentration $1x10^{-4}$ M) in buffer (0.5 M phosphate, pH=8) for 5 minutes at 25°C prior to addition of reagent and substrate. Activity remaining in samples pre-incubated with iso-OMPA was attributed to AChE. Activities in plasma and brain were collected over 2 and 6 minute run times, respectively, with readings every 12 seconds. An analytical AChE standard was prepared from the membranes of equine red blood cells in general accordance with the method developed for human red blood cells by Dodge et al. (1963) as modified by Hansen and Wilson (1999). This standard was run with all plasma samples assayed. A second analytical standard was prepared from the pooled brains of several unexposed quail consistent with the brain sample preparation methods described above. Both analytical standards were run with all brain samples assayed. All measures were made in triplicate. Activity in a sample was the mean of at least 2 of the 3 measures obtained. One of the triplicate measures of sample activity was rejected in cases where it differed from both of the others by >5% while the others differed by <5%.

3.2.2.4 Reconstruction of Exposure and Toxicity

Recovery from inhibition of ChE activity in plasma and brain at 24 to 72 hours post-exposure was examined for each OP. Comparisons were made between the PC groups, all alimentary exposures, and the FO groups from the simulated field, for which exposure route is to be inferred.

3.2.3 Statistics

Data in all analyses were tested for normality and homogeneity of variance. Observations were examined using analysis of variance (ANOVA) to test for main and interaction effects. Post hoc means tests were completed to differentiate between treatment levels using Tukey's HSD method. Select comparisons between groups were made using independent samples t tests. Significance was associated with $p \leq 0.05$. Findings approaching significance ($0.10 \geq p > 0.05$) are also reported. All analyses were run on PASW Statistics 18 (IBM, Chicago, IL, USA).

3.3 RESULTS

3.3.1 Pilot Studies

Spray rate-dependent inhibition of quail plasma ChE activity at 24 hours post-exposure was evident in pilot study sprays with all compounds. One way ANOVA revealed significant effects of application rate for each of DCP (F_2=4.992, p=0.029), ETP (F_2=6.524, p=0.018), MMP (F_2=9.966, p=0.003) and NLD (F_2=23.766, p<0.001).

Ordinary least squares (OLS) linear regression revealed significant relationships between pesticide concentrations in sprays and inhibition of plasma ChE activity at 24 hours post-exposure (Figure 3.2A to 3.2D)(DCP, F_1=10.883, p=0.006; ETP, F_1=14.493, p=0.003; MMP,

F_1=21.484, p<0.001; NLD, F_1=50.542, p<0.001). The observed relationships between compound application rates and inhibition of quail plasma ChE activity were used to estimate the spray rates required for 60% inhibition of plasma ChE activity. These rates were 2550, 1310, 7800, and 33000 g compound/ha for DCP, ETP, MMP, and NLD, respectively.

3.3.2 Acetylcholinesterase Activity in Plasma

The contribution of AChE to total plasma ChE activity was investigated in plasma from 48 quail later submitted to simulated field exposures with DCP. No AChE activity was detected in half of the quail plasma samples analyzed. Low levels of AChE activity were reported by the microplate reader in the other half of the samples (maximum plasma AChE activity=0.12 mol substrate hydrolyzed/min./mL). However, inspection of the plots generated by the microplate reader software revealed non-linear relations. Further, a microplate in which activity had been reported was left to stand for 24 hours at room temperature following assay. No visible yellow colour developed in wells containing samples while wells containing standards developed a deep yellow hue. No AChE activity was apparent in quail plasma.

3.3.3 Pre-exposure plasma

Significant effects appeared in the analysis of pre-exposure plasma data. These effects were not apparent during assays due to the natural high variability in plasma ChE activity because these effects were small relative to the effects in post-exposure plasma samples analyzed during the same period. However, these effects became apparent once statistical analyses of pre-exposure plasma data were completed. They were then investigated.

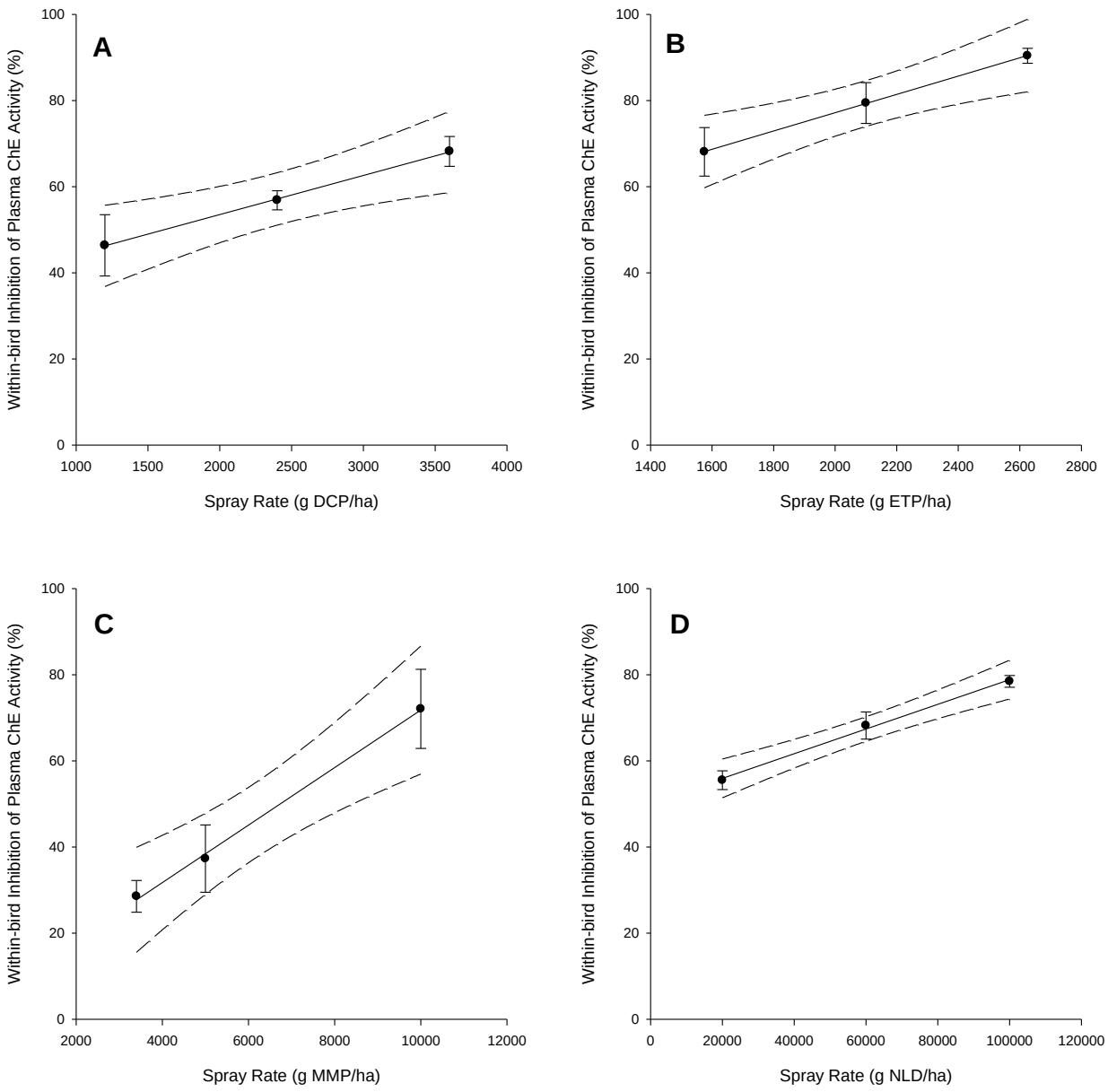


Figure 3.2. Linear variation in plasma cholinesterase (ChE) activity in quail with different concentrations of the active ingredient used in four pilot study sprays each performed with a commercial formulation of a different organophosphate (OP) pesticide where the active ingredient and its commercial formulation are: **A**) dicrotophos (DCP) in BIDRIN® 8; **B**) ethoprop (ETP) in MOCAP®; **C**) methamidophos (MMP) in MONITOR®; and **D**) naled (NLD) in DIBROM®. Dashed lines denote 95% confidence intervals for the linear relations illustrated. Sample size was 5 in each group. Error bars denote (+/-) standard error.

Effects in pre-exposure plasma ChE activity data were characterized using ANOVA. Firstly, a 4 factor mixed-model ANOVA (OP by Treatment by Time by Replicate) was conducted using the entire pre-exposure plasma ChE activity data set (N=593). This ANOVA revealed significant effects of OP and OP*Replicate (Table 3.2). Data in the ANOVA were pooled first under Treatment and then under Time with similar findings. Effects found in the final 2 factor mixed-model ANOVA were those of OP and OP*Replicate (Table 3.2). These results were examined graphically (Figure 3.3A). The relationship between the factors OP and Replicate appeared random, explaining the absence of any significant main effect of Replicate. These same data were then pooled under Replicate and examined with a 3 factor fixed-model ANOVA (OP by Treatment by Time). The only significant effect observed was that of OP (F_3=54.54, p<0.001). No other main or interaction effects approached the significant level.

Table 3.2. Results (F statistic, p value) of the analysis of variance (ANOVA) of pre-exposure plasma cholinesterase activity data for the entire data set (all treatments) and for simulated field data alone. Factors are Organophosphate (OP) insecticide, Treatment (Tr), Time (Ti), and Replicate (Rep). Results that did not approach the significant level (p>0.10) are either omitted or not reported (-).

Results (F statistic, p value) from Analysis of Pre-exposure Plasma Data					
Data from All Treatments			Data from Simulated Field Treatments		
ANOVA Model	Source of Variability	Results	ANOVA Model	Source of Variability	Results
4 Factor Mixed	OP	6.61, 0.025	4 Factor Mixed	OP	6.47, 0.026
	Tr	-		Tr	-
	Ti	-		Ti	-
	Rep	-		Rep	-
	OP*Rep	9.54, 0.008		OP*Rep	8.19, 0.055
2 Factor Mixed	OP	6.59, 0.025	2 Factor Fixed	OP	36.07, <0.001
	Rep	-		Tr	-
	OP*Rep	9.39, <0.001		OP*Tr	-

The lab in which buffers and reagents were prepared and maintained and the lab which contained the microplate reader were both located in older wings of the Toxicology Centre known for their aged climate control systems. A thermocouple thermometer (Sper Scientific Inc. Model 800005, Scottsdale, AZ, USA) was used to obtain measures of temperature within the work space and check the calibration of the microplate reader's incubator during the period in which brain ChE assays were performed. Temperature in the lab where ChE assay buffers and reagent solutions were prepared and maintained varied between 2°C and 28°C through the period of monitoring. The actual microplate reader incubator temperature was consistently more than 3°C lower than the temperature reported by the instrument itself (i.e. actual assay temperature <22°C while the microplate reader reported 25°C in the incubator).

OLS regression was used to determine the extent to which ambient temperature influenced pre-exposure plasma ChE data. Ambient temperatures recorded at the local airport by Environment Canada for the date and time at which pre-exposure plasma ChE assays were completed (www.climate.weatheroffice.gc.ca/climateData) significantly explained some of the variation in pre-exposure plasma ChE activity (F_1=27.269, $p<0.001$, r^2=0.124) for assays completed during times when ambient temperatures exceeded 22°C but not for assays completed during times when ambient temperatures were lower than 22°C. However, the residuals in the significant regression were not normally distributed.

An OLS regression using ChE activity in the analytical standard to predict ChE activity in the unknowns for ambient temperatures exceeding 22°C was also significant (F_1=6.897, p=0.006) but it did not explain as much of the variation in ChE activity in quail plasma unknowns (r^2=0.035) as did ambient temperature.

Pre-exposure plasma ChE activity data from the simulated field (FO, FD, and FS collectively) and control samples (NC and PC individually) were subject to ANOVA to assess if effects in pre-exposure data differed among these groups. A 4 factor mixed-model ANOVA of data from the simulated field (OP by Treatment (FO, FD, and FS only) by Time by Replicate) revealed only a significant effect of OP and an effect of the OP*Replicate interaction that approached the significant level (Table 3.2). These simulated field data were then pooled first under Replicate and then under Time with similar results. The only significant effect in the final 2 factor fixed-model ANOVA (OP by Treatment (FO, FD, and FS only)) was that of OP (Table 3.2). These ANOVA results were examined graphically (Figure 3.3B) for comparison with the 2 factor mixed-model ANOVA (OP by Replicate) with the entire data set (Figure 3.3A). Temperature-related effects appeared relatively absent from analysis of data obtained on the simulated field within each of the OPs although comparisons between OPs remained compromised. A 3 factor mixed-model ANOVA of NC data (OP by Time by Replicate) lacked significant effects although the effects of OP and the OP*Replicate interaction were those closest to the significant level (Table 3.3). These NC data were pooled first under Replicate and then under Time with similar results. The effects observed of OP in the final one-way ANOVA were significant (Table 3.3). Similarly, a 3 factor mixed-model ANOVA of PC data (OP by Time by Replicate) lacked significant effects with only the effect of OP and the OP*Replicate interaction approaching the significant level (Table 3.3). Pooling of data resulted in a final one-way ANOVA revealing a significant effect of OP (Table 3.3). Further, the effects evident in pre-exposure NC and PC data appeared to be related as OLS regression revealed a significant and well-correlated relationship between mean plasma ChE activities within OPs between the two data sets (F_1=28.06, p=0.034, r^2=0.933, slope=1.067).

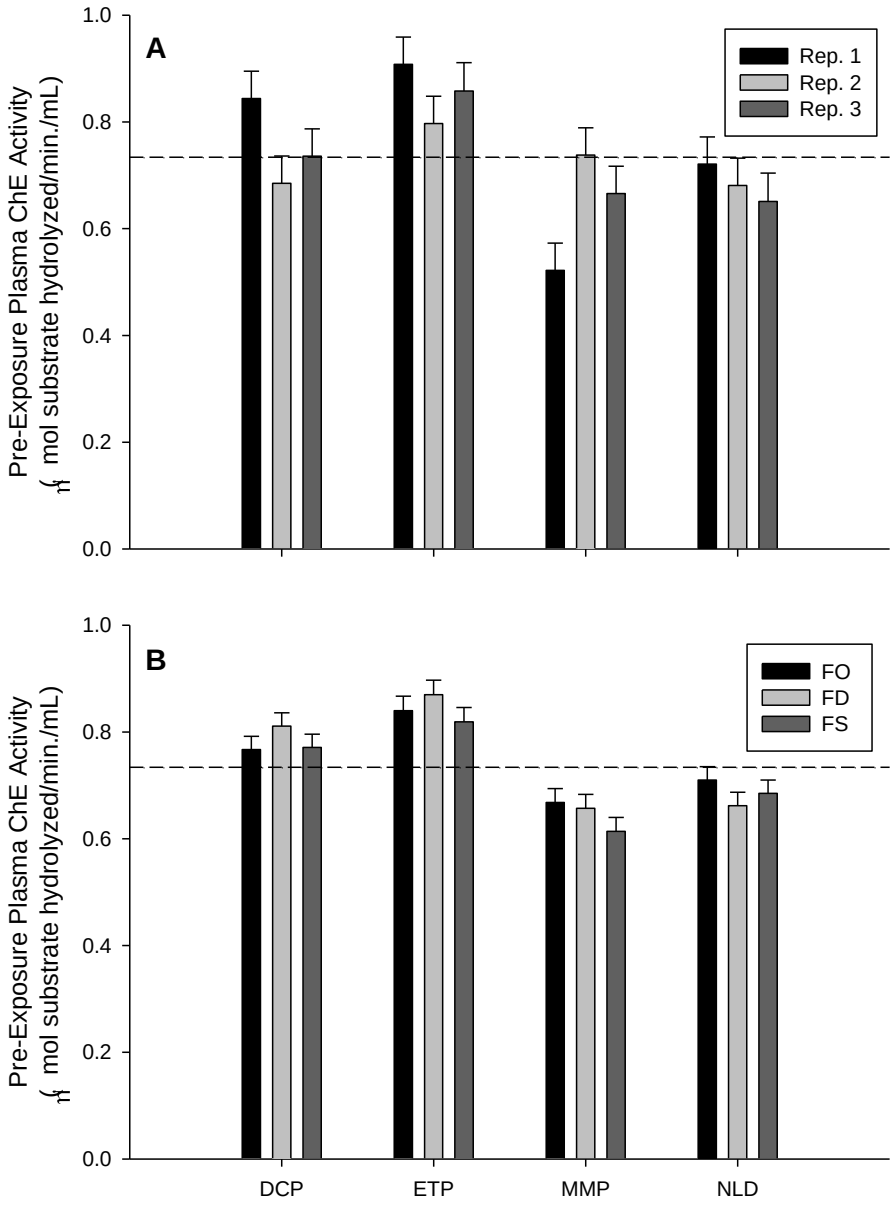


Figure 3.3. Variation in pre-exposure plasma cholinesterase (ChE) activity in quail within different combinations of experimental factors where these factor combinations are: **A**) Organophosphate (OP) insecticide and Replicate; and **B**) OP and Treatment (only the three levels on the simulated field; field only (FO), field plus diet (FD), and field plus spray (FS)). The dashed lines denote the grand mean pre-exposure plasma ChE activity (0.734 mol substrate hydrolyzed/min./mL). Error bars denote (+/-) standard error. Statistically significant effects include those of OP ($p=0.025$) and the OP by Replicate interaction ($p<0.001$) in **A**) and only that of OP ($p<0.001$) in **B**).

Table 3.3. Results (F statistics, p value) of the analysis of variance (ANOVA) of pre-exposure plasma cholinesterase activity data for control groups (negative, positive). Factors are Organophosphate (OP) insecticide, Time (Ti), and Replicate (Rep). Results that did not approach the significant level (p>0.10) are either omitted or not reported (-).

ANOVA Model	Source of Variability	Results (F statistic, p value) from Analysis of Pre-exposure Plasma Data in Control Groups	
		Negative	Positive
3 Factor Mixed	OP	3.46, 0.093	3.29, 0.100
	Ti	-	-
	Rep	-	-
	OP*Rep	3.01, 0.103	4.12, 0.054
1 Factor	OP	9.07, <0.001	12.19, <0.001

Measures of effect size (eta-squared, η^2) were used to assess the contributions of different sources (i.e. error, factors, and interactions) to variability within the four different factorial fixed-model ANOVAs produced in the analysis of pre-exposure plasma ChE activity data: 1) the 3 factor ANOVA of all data (OP by Treatment by Time); 2) the 3 factor ANOVA of data from the simulated field (OP by Treatment (FO, FD, and FS only) by Time); the 2 factor ANOVA of NC data (OP by Time); and 4) the 2 factor ANOVA of PC data (OP by Time). Regardless of the ANOVA considered, error accounted for approximately three-quarters of the variability in the models ($0.732 \leq \eta^2 \leq 0.775$), OP accounted for approximately one-fifth of the variability in the models ($0.193 \leq \eta^2 \leq 0.241$), and the remaining factors and interactions collectively accounted for less than 4% of variability in the models ($\sum\eta^2<0.04$) (Table 3.4). Further, the NC and PC treatments comprised approximately 40% of the data but accounted for approximately 47% of both variability and error in the models.

The minimum and maximum mean (95% CI) pre-exposure plasma ChE activities within the various combinations of OP, Treatment, and Time were 0.611 (0.537-0.685)(MMP, FS, 24 hr.; n=15) and 0.901 (0.813-0.989)(ETP, NC, 24 hr.; n=14) µmol substrate hydrolyzed/min./mL,

respectively. Pooling of all data resulted in a pre-exposure grand mean (SE) plasma ChE activity of 0.734 (0.007, N=593) µmol substrate hydrolyzed/min./mL.

3.3.4 Post-exposure Plasma

A 4 factor mixed-model ANOVA (OP by Treatment by Time by Replicate) was performed with all post-exposure plasma ChE activity data (N=595). The analysis revealed significant main effects of Treatment and Time and significant interaction effects OP*Treatment, Treatment*Time, and OP*Treatment*Time*Replicate while the main effect of OP approached the significant level (Table 3.5). Measures of effect size, partial eta-squared (η_p^2), were used to compare the significant main and interaction effects. The effect of the final interaction term involving Replicate was very small relative to effects of the other sources as it explained less than 9% of within-source variability (η_p^2=0.089) while other sources explained approximately 64% to >99% of within-source variability ($0.641 \leq \eta_p^2 \leq 0.999$). The significant effect of the final interaction term was deemed too small to be either of interest or concern and data were pooled under Replicate. The resulting 3 factor fixed-model ANOVA of all post-exposure data (N=595) revealed significant effects of all sources (Table 3.5). Relative to the analogous ANOVA with pre-exposure data, the introduction of effects from the actual exposures increased variability in the ANOVA by greater than two-fold (pre-exposure total sum of squares (SS)=16.756, post-exposure total SS=37.495) while error was reduced marginally (pre-exposure error SS=12.474, post-exposure error SS=10.978). Here error explained less than 30% of variability in the post-exposure ANOVA (η^2=0.293). This ANOVA was compared to the analogous pre-exposure plasma ChE activity ANOVA using η_p^2 (Table 3.4). Although the actual exposures greatly

Table 3.4. Variability (sum of squares (SS)) and measures of effect size (eta-squared (η^2) and partial eta-squared (η_p^2)) in fixed-model ANOVA of pre-exposure and post-exposure plasma cholinesterase activity data. Information is provided for the entire data set (All) and for portions of the data set including data from treatments on the simulated field collectively (Field) and for control data individually (negative control (NC) and positive control (PC)).

Data Input	Analysis	Period							
		Pre-exposure				Post-exposure			
		Source	SS	η^2	η_p^2	Source	SS	η^2	η_p^2
All	ANOVA	Total	16.756			Total	37.495		
		OP	3.697	0.221	0.229	OP	2.573	0.069	0.190
		Treatment	0.083	0.005	0.007	Treatment	12.731	0.340	0.537
		Time	0.018	0.001	0.001	Time	6.828	0.182	0.383
		OP*Tr.	0.196	0.012	0.015	OP*Tr.	1.831	0.049	0.143
		OP*Ti.	0.017	0.001	0.001	OP*Ti.	0.279	0.007	0.025
		Tr.*Ti.	0.034	0.002	0.003	Tr.*Ti.	1.735	0.046	0.136
		OP*Tr.*Ti.	0.238	0.014	0.019	OP*Tr.*Ti.	0.540	0.014	0.047
		Error	12.474	0.744		Error	10.978	0.293	
Field	ANOVA	Total	8.984			Total	16.037		
		OP	2.121	0.236	0.244	OP	3.487	0.217	0.398
		Treatment	0.047	0.005	0.007	Treatment	0.659	0.041	0.111
		Time	0.003	<0.001	<0.001	Time	6.604	0.378	0.535
		OP*Tr.	0.094	0.010	0.014	OP*Tr.	0.185	0.012	0.034
		OP*Ti.	0.019	0.002	0.003	OP*Ti.	0.254	0.016	0.046
		Tr.*Ti.	0.004	0.000	0.001	Tr.*Ti.	0.010	0.001	0.002
		OP*Tr.*Ti.	0.120	0.013	0.018	OP*Tr.*Ti.	0.104	0.006	0.019
		Error	6.577	0.732		Error	5.275	0.329	
NC	ANOVA	Total	3.919			Total	4.170		
		OP	0.755	0.193	0.199	OP	0.217	0.052	0.054
		Time	0.045	0.011	0.015	Time	<0.001	<0.001	<0.001
		OP*Ti.	0.080	0.020	0.026	OP*Ti.	0.156	0.037	0.039
		Error	3.038	0.775		Error	3.796	0.910	
PC	ANOVA	Total	3.815			Total	4.876		
		OP	0.920	0.241	0.244	OP	0.942	0.193	0.462
		Time	<0.001	<0.001	<0.001	Time	2.383	0.489	0.685
		OP*Ti.	0.037	0.010	0.013	OP*Ti.	0.453	0.093	0.292
		Error	2.858	0.749		Error	1.097	0.225	

increased variability, the effect size associated with OP decreased slightly from the pre-exposure ANOVA (η_p^2=0.229) to post-exposure ANOVA (η_p^2=0.190). Instead the effects of the actual exposures were manifest largely in other main effects and the interactions. Effect sizes in Treatment, Time, OP*Treatment, OP*Time, Treatment*Time, and OP*Treatment*Time as measured using η_p^2 increased by approximately 77-fold, 383-fold, 10-fold, 25-fold, 45-fold, and more than 2-fold, respectively, from pre-exposure to post-exposure (Table 3.4).

Table 3.5. Results (F statistic, p value) of the analysis of variance (ANOVA) of post-exposure plasma cholinesterase activity data for the entire data set (all treatments) and for simulated field data alone. Factors are Organophosphate insecticide (OP), Treatment (Tr), Time (Ti), and Replicate (Rep). Results that did not approach the significant level ($p>0.10$) are either omitted or not reported (-).

Results (F statistic, p value) from Analysis of Post-exposure Plasma Data					
Data from All Treatments			Data from Simulated Field Treatments		
ANOVA Model	Source of Variability	Results	ANOVA Model	Source of Variability	Results
4 Factor Mixed	OP	4.73, 0.051	4 Factor Mixed	OP	4.92, 0.047
	Tr	64.62, <0.001		Tr	40.30, 0.002
	Ti	1715, <0.001		Ti	802.77, 0.001
	Rep	-		Rep	-
	OP*Tr	3.57, 0.004		OP*Tr	3.95, 0.021
	OP*Rep	-		OP*Rep	3.42, 0.076
	Tr*Ti	24.15, <0.001		Tr*Ti	-
	OP*Tr*Ti	-		OP*Tr*Ti	2.87, 0.057
	OP*Ti*Rep	-		OP*Ti*Rep	10.82, <0.001
	OP*Tr*Ti*Rep	1.95, 0.005		OP*Tr*Ti*Rep	-
3 Factor Fixed	OP	43.36, <0.001	3 Factor Fixed	OP	73.37, <0.001
	Tr	160.92, <0.001		Tr	20.80, <0.001
	Ti	345.22, <0.001		Ti	382.84, <0.001
	OP*Tr	7.72, <0.001		OP*Tr	1.94, 0.074
	OP*Ti	4.70, 0.003		OP*Ti	-
	Tr*Ti	21.93, <0.001		Tr*Ti	-
	OP*Tr*Ti	2.28, 0.008		OP*Tr*Ti	-

Post-exposure plasma ChE activity data from the simulated field (FO, FD, and FS collectively) and controls (NC and PC individually) were each subject to ANOVA. A 4 factor mixed-model ANOVA of post-exposure data from the simulated field (OP by Treatment (FO, FD, and FS only) by Time by Replicate) revealed significant main and interaction effects of OP, Treatment, Time, OP*Treatment, and OP*Time*Replicate and interaction effects of OP*Replicate and OP*Treatment*Time that approached the significant level Table 3.5). Interaction effects involving Replicate were ignored here specifically so that data could be pooled under Replicate and a 3 factor fixed-model ANOVA of simulated field data could be performed and compared with the analogous ANOVA performed with pre-exposure data from

the simulated field. This ANOVA of post-exposure plasma data from the simulated field revealed significant effects of OP, Treatment, Time, and OP*Time and an OP*Treatment interaction effect that approached the significant level (Table 3.5). Variability, error, and effect sizes (both η^2 and η_p^2) in this ANOVA were compared with those of the analogous pre-exposure simulated field data ANOVA. Variability almost doubled (pre-exposure total SS=8.984, post-exposure total SS=16.037) while error diminished slightly (pre-exposure error SS=6.577, post-exposure error SS=5.275). Here error explained about one-third of variability in the post-exposure ANOVA (η^2=0.329), much less than in the pre-exposure ANOVA. Time explained approximately 38% of variability (η^2=0.378), whereas it explained almost none of the variability in the pre-exposure ANOVA. OP explained approximately 22% of the variability (η^2=0.217), similar to that of the pre-exposure ANOVA. Comparisons of η_p^2 between the two analogous ANOVAs revealed an increase in variability associated with OP and other sources (Table 3.4). Effect sizes in OP, Treatment, Time, OP*Treatment, OP*Time, and Treatment*Time as measured using η_p^2 increased by approximately 1.6-fold, 16-fold, 500-fold, 2.4-fold, 15-fold, and 2-fold, respectively, from pre-exposure to post-exposure (Table 3.4).

Post-exposure NC plasma ChE activity data were analyzed in the same manner. No significant effects were found in any of the post-exposure ANOVAs. Data were pooled into a single set having mean (SE) plasma ChE activity of 0.828 (0.017)(n=119) µmol substrate hydrolyzed/min./mL. Comparisons of variability and effect size (both both η^2 and η_p^2) in the analogous 2 factor fixed-model ANOVAs (OP by Time) using pre-exposure and post-exposure data revealed that the post-exposure ANOVA contained marginally more variability (pre-exposure total SS=3.919, post-exposure total SS=4.170) but 25% more error (pre-exposure error SS=3.038, post-exposure error SS=3.796). As a result, error explained 91% of variability in the

post-exposure ANOVA (η^2=0.910). The decrease in η_p^2 associated with OP from pre-exposure ANOVA to post-exposure ANOVA (Table 3.4) is consistent with the lack of a significant effect of OP in the post-exposure analysis.

A 3 factor mixed-model ANOVA (OP by Time by Replicate) of post-exposure PC plasma ChE activity data revealed significant effects of Time and the final interaction of term of OP*Time*Replicate as well an OP*Time interaction that approached significance (Table 3.6). Inspection of the marginal means revealed a very weak response of plasma ChE activity to the oral dose in the third ETP replicate and a similar weak response was also eventually found in observations in brain ChE activity in the same replicate (reported later). Data from this replicate were deleted and the resulting fractional three factor mixed-model ANOVA was free of significant effects involving the factor Replicate. The data were then pooled under Replicate with the resulting two factor fixed-model ANOVA showing significant effects of OP, Time, and OP*Time interaction (Table 3.6). Mean (SE) plasma ChE activities in quail dosed orally with DCP, ETP, MMP, and NLD were 0.323 (0.027, n=15), 0.051(0.035, n=9), 0.491 (0.027, n=15), and 0.488 (0.027, n=15) µmol substrate hydrolyzed/min./mL, respectively, at 24 hours post-exposure and were 0.627 (0.027, n=15), 0.587 (0.033, n=10), 0.695 (0.027, n=15), and 0.671 (0.027, n=15) µmol substrate hydrolyzed/min./mL, respectively, at 72 hours post-exposure. Variability and effect size in the analogous 2 factor fixed-effects ANOVAs (OP by Time) using pre-exposure and post-exposure PC data sets were compared. Total variability increased by more than a quarter (pre-exposure total SS=3.815, post-exposure total SS=4.876) while the variability attributed to error declined by more than half (pre-exposure error SS=2.858, post-exposure error SS=1.097). Here error explained approximately 23% of variability while the effects of OP, Time, and their interaction explained approximately 19%, 49%, and 9% of variability, respectively

(Table 3.4). Effect sizes in OP, Time, and OP*Time as measured using η_p^2 increased by approximately 2-fold, 685-fold, and 22-fold, respectively, from pre-exposure to post-exposure (Table 3.4).

Table 3.6. Results (F statistics, p value) of the analysis of variance (ANOVA) of post-exposure plasma cholinesterase activity data for control groups (negative, positive). Factors are Organophosphate (OP) insecticide, Time (Ti), and Replicate (Rep). Results that did not approach the significant level (p>0.10) are either omitted or not reported (-).

ANOVA Model	Source of Variability	Results (F statistic, p value) from Analysis of Post-exposure Plasma Data in Control Groups	
		Negative Control	Positive Control
3 Factor Mixed	OP	-	-
	Ti	-	168.64, 0.006
	Rep	-	-
	OP*Ti	-	3.70, 0.081
	OP*Ti*Rep	-	2.93, 0.012
2 Factor Fixed	OP	-	28.90, <0.001
	Ti	-	219.39, <0.001
	OP*Ti	-	13.91, <0.001

Post-exposure plasma ChE activity data from the simulated field were analyzed further on an OP by OP basis. Three factor mixed-model ANOVA (Time x Treatment (FO, FD, and FS only) x Replicate) of DCP data revealed significant effects of Time, Treatment, and the Time*Replicate interaction (Table 3.7). Inspection of the data showed that the response varied among replicates, being consistent among first two but relatively large in the third. Elimination of data in the third replicate resulted in a fractional three factor mixed-model ANOVA that was free of significant effects involving the factor Replicate. These data were then pooled under Replicate with the resulting two factor fixed-model ANOVA showing significant effects of Time and Treatment (Table 3.7). *Post hoc* tests of Treatment effects using data pooled under Time

revealed that the FS treatment was significantly different from either the FO or FD (Tukey HSD, $p<0.001$). Mean (SE) plasma ChE activities in quail in FO, FD, and FS treatments were 0.281 (0.027, n=10), 0.296 (0.027, n=10), and 0.179 (0.027, n=10) µmol substrate hydrolyzed/min./mL, respectively, at 24 hours post-exposure and were 0.532 (0.027, n=10), 0.546 (0.027, n=10), and 0.390 (0.027, n=10) µmol substrate hydrolyzed/min./mL, respectively, at 72 hours post-exposure.

Three factor mixed-model ANOVA of ETP data revealed significant effects of Time and Treatment and an effect in the Treatment*Replicate interaction that approached significance (Table 3.7). The interaction term involving Replicate was deemed too small to be either of interest or concern. The ANOVA revealed the variability associated with the interaction hypothesis (SS=0.079) was a fraction of that associated with the hypotheses of either Treatment (SS=2.409) or Time (SS=0.438). Data were pooled under Replicate with the resulting two factor fixed-model ANOVA showing significant effects in Time and Treatment (Table 3.7). *Post hoc* tests of Treatment effects using data pooled under Time revealed that the FS treatment was significantly different from both the FO and FD (Tukey HSD, $p\leq0.002$). Mean (SE) plasma ChE activities in quail in FO, FD, and FS treatments were 0.450 (0.033, n=15), 0.406 (0.033, n=15), and 0.250 (0.034, n=14) µmol substrate hydrolyzed/min./mL, respectively, at 24 hours post-exposure and were 0.754 (0.033, n=15), 0.717 (0.033, n=15), and 0.623 (0.033, n=15) µmol substrate hydrolyzed/min./mL, respectively, at 72 hours post-exposure.

Three factor mixed-model ANOVA of MMP data revealed significant effects of Time, Treatment, and the Time*Replicate interaction (Table 3.7). Here the interaction Time*Replicate contributed almost as much variability to the ANOVA (SS=0.113) as did Treatment (SS=0.133)

Table 3.7. Results (F statistic, p value) of the analysis of variance (ANOVA) of post-exposure plasma cholinesterase data within levels of the factor Organophosphate insecticide, those being dicrotophos (DCP), ethoprop (ETP), methamidophos (MMP), and naled (NLD). Factors within the ANOVA are Treatment (Tr), Time (Ti), and Replicate (Rep). Results that did not approach the significant level (p>0.10) are either omitted or not reported (-).

ANOVA Model	Source of Variability	Results (F statistic, p value) from Analysis of Post-exposure Plasma Data			
		DCP	ETP	MMP	NLD
3 Factor Mixed	Tr	61.97, 0.001	11.14, 0.023	11.31, 0.022	4.54, 0.094
	Ti	20.73, 0.045	129.79, 0.008	34.40, 0.028	12.76, 0.070
	Rep	-	-	-	14.60, 0.015
	Tr*Rep	-	4.29, 0.094	-	-
	Ti*Rep	9.27, 0.031	-	8.76, 0.034	-
2 Factor Fixed	Tr	16.09, <0.001	13.02, <0.001	4.07, 0.021	-
	Ti	119.72, <0.001	142.76, <0.001	119.27, <0.001	74.59, <0.001

while the contribution of Time was more than an order of magnitude greater (SS=1.948). Inspection of marginal means and further analysis revealed that results in Replicate 1 were responsible for the Time*Replicate interaction. Quail in Replicate 1 had the lowest plasma ChE activity at 24 hours post-exposure but the highest activity at 72 hours post-exposure (i.e. the greatest recovery of activity from 24 to 72 hour post-exposure). This difference was sufficient to produce the significant interaction but not particularly troubling in interpretation. All data were retained and pooled under Replicate. The resulting two factor fixed-model ANOVA showing significant effects of Time and Treatment (Table 3.7). *Post hoc* tests of Treatment effects using data pooled under Time revealed that the FS treatment was significantly different from the FD (Tukey HSD, p=0.029) but not the FO (Tukey HSD, p=0.151). Mean (SE) plasma ChE activities in quail in FO, FD, and FS treatments were 0.444 (0.033, n=15), 0.428 (0.033, n=15), and 0.341 (0.034, n=14) µmol substrate hydrolyzed/min./mL, respectively, at 24 hours post-exposure and were 0.690 (0.033, n=15), 0.755 (0.033, n=15), and 0.658 (0.033, n=15) µmol substrate hydrolyzed/min./mL, respectively, at 72 hours post-exposure.

Three factor mixed-model ANOVA of NLD data revealed a significant effect of the Time*Replicate interaction and trends towards effects in Time and Treatment (Table 3.7). Variability associated with the interaction (SS=0.173) exceeded that of Treatment (SS=0.041) but was smaller than that of Time (SS=1.102). Inspection of marginal means revealed that although each replicate showed recovery of plasma ChE activity from 24 to 72 hours post-exposure, these recoveries varied among replicates. This variation was sufficient to produce a significant interaction effect but not troubling in interpretation. Further analyses revealed that omission of data from Replicate 3 reduced the interaction effect to less than significant but also reduced the effects of Time and Treatment. Further, when these data were pooled under Replicate and subject to fixed-model ANOVA, the results were consistent with the analogous ANOVA using all data but the ANOVA with all data performed better (data set lacking Replicate 3, ANOVA r^2=0.376; all data, ANOVA r^2=0.456). The significant interaction was ignored and analysis proceeded using all data pooled under Replicate. Two factor fixed-model ANOVA of pooled data revealed only a significant effect of Time (Table 3.7). Further, no significant effect of Treatment was observed when data from 24 and 72 hour post-exposure periods were analyzed separately using one-way ANOVA. Mean (SE) plasma ChE activities in quail in FO, FD, and FS treatments were 0.365 (0.031, n=15), 0.358 (0.031, n=15), and 0.295 (0.031, n=15) µmol substrate hydrolyzed/min./mL, respectively, at 24 hours post-exposure and were 0.593 (0.031, n=15), 0.528 (0.031, n=15), and 0.561 (0.031, n=15) µmol substrate hydrolyzed/min./mL, respectively, at 72 hours post-exposure.

3.3.5 Brain

ChE activity in the brain samples was far more sensitive to temperature fluctuations than the activity in either of the equine RBC AChE standard or the quail plasma BChE. Numerous comparisons of repeat batches of brain ChE assays conducted on the same unknowns, but on different days and with different buffers and reagents, always revealed a strong correlation between the two sets of data. Ordinary least squares (OLS) regression was used to investigate the relationship between the batches of assays. However, as the intercept was never significant in these OLS regressions and as it seemed logical that the regression line pass through the origin, regression through the origin (RTO) was thereafter used to explore the relationship between the two sets of assay results. RTO using two batches of brain ChE assay that appeared to differ greatly revealed a significant relationship between them (F_1=4307.185, $p<0.001$) in which variation in one data set explained greater than 99% of the variation about the origin of the other data set (r^2=0.997)(Figure 3.4A). However, the slope of the RTO line (slope=1.373, t=65.629, $p<0.001$) varied from the value expected of repeat analyses of a single sample set (i.e. slope=1). These results indicate that the difference in the measured activity in an unknown between the two assays was proportional to the measured activity in the unknown itself in either assay. One of the data points with relatively high activity (Figure 3.4A, open circle, x=7.066 µmol substrate hydrolyzed/min./g wet mass, y=9.703 µmol substrate hydrolyzed/min./g wet mass) was then chosen as a surrogate analytical standard of assumed true activity equal to 8.000 µmol substrate hydrolyzed/min./g wet mass and ChE activity in the other unknowns of both data sets were standardized to the assumed true activity in the surrogate analytical standard using Eq. 3.1:

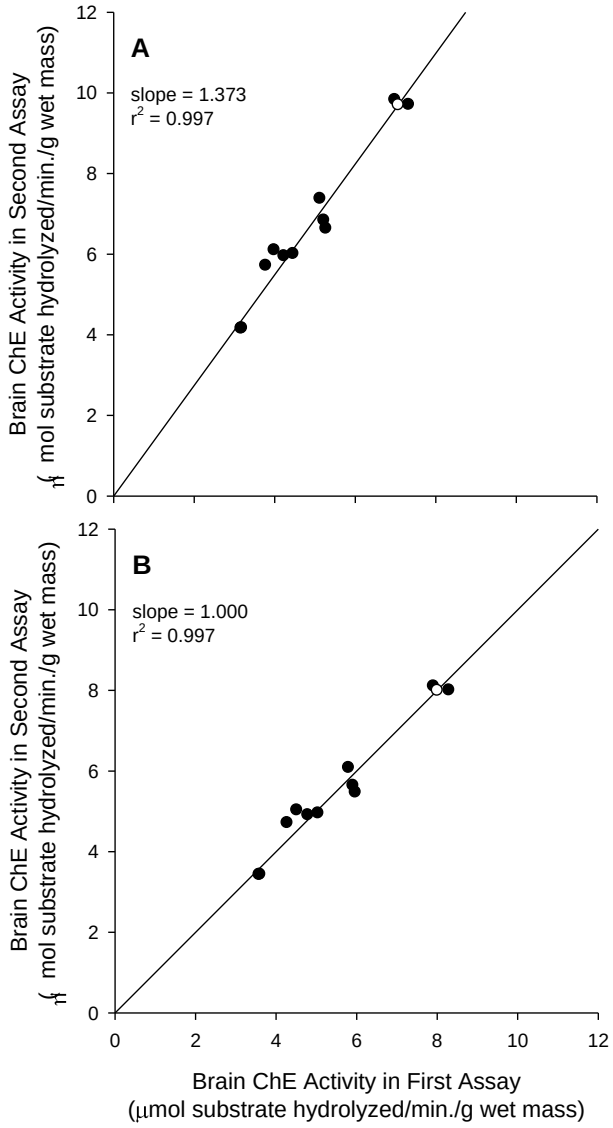


Figure 3.4. Regression through the origin between two different batches of brain cholinesterase (ChE) activity assays conducted at different temperature regimes but using the same brain sample preparations from 11 quail having varying degrees of inhibition of brain ChE activity. The regression using raw data (**A**) is significant and illustrates how the results from the two different batches of assays are highly correlated and proportional but not identical due to differences in the temperature regimes. The regression using standardized data (**B**) is also significant and illustrates how standardization of the data based upon an analytical standard (open circle) having an assumed 'true' ChE activity of 8.000 μmol substrate hydrolyzed/min./g wet mass brain maintains both the relationship and correlation structure while eliminating differences in brain ChE activity in the other samples (solid circles) between batches of assays due to differences in temperature regimes.

$$Unk_{std} = Std_{true}/Std_{meas} \times Unk_{meas} \quad \text{(Eq. 3.1)}$$

where Unk_{std} = standardized ChE activity of the unknown

Std_{true} = true ChE activity of the surrogate analytical standard

Unk_{meas} = measured ChE activity of the unknown, and

Std_{meas} = measured ChE activity of the surrogate analytical standard.

The resulting standardized data sets were again subjected to RTO (Figure 3.4B). The relationship between the two data sets was again significant (F_1=4310.244, $p<0.001$) and the correlation structure was maintained (r^2=0.997) while the slope of the regression line became unity (slope=1.000, t=65.652, $p<0.001$). These findings led to the development of an analytical standard derived from brains of quail neither exposed to OPs nor used within the experiment. This avian brain ChE analytical standard was run concurrently with all brain unknowns. A total of 123 analytical standards were run for the 593 brain samples assayed (approx. 5 samples/standard). The distribution of observed ChE activity in the standard was multimodal (kurtosis=-1.586) and possessed a wide range (5.11 µmol substrate hydrolyzed/min./g wet mass). The mean and median ChE activities of the standard were 10.66 and 10.35 µmol substrate hydrolyzed/min./g wet mass, respectively. The median activity in the standard was assumed to be a more robust indicator of central tendency than the mean and was designated as the true activity of the analytical standard. The measured activity in all brain ChE unknowns was standardized to the median analytical standard activity using Eq. 3.1 prior to analysis. Thereafter analyses proceeded on a treatment by treatment basis.

Analysis of variance of standardized NC brain ChE data revealed neither main effects nor any interactions. Pooled standardized NC data failed the Shapiro-Wilk's normality test (p=0.012)

and inspection of the data revealed three potential outliers having values greater than 3 standard deviations from the mean. Omission of these potential outliers resulted in a data set that passed tests of normality. Mean (SD) NC brain ChE activities with and without potential outliers were 7.80 (0.62) (n=113) and 7.83 (0.57) (n=110) µmol substrate hydrolyzed/min./g wet mass, respectively.

Three factor mixed model ANOVA (OP x Time x Replicate) of PC brain ChE data revealed significant effects of OP (F_3=7.54, p=0.019), Time (F_1=363.35, p=0.003), OP*Time (F_3=12.24, p=0.006), and OP*Replicate (F_6=39.74, p<0.001). Inspection of the marginal means revealed a cause for the significant OP*Replicate interaction. Results with ETP and MMP both contained anomalous responses in their third replicates, suggesting error in the oral doses given to PC quail. Exclusion of data from the third replicates of both ETP and MMP produced ANOVA results showing significant effects of OP (F_3=153.03, p<0.001) and Time (F_1=683.92, p=0.005) and a trend towards an OP*Time interaction (F_3=5.75, p=0.062) while eliminating the OP*Replicate interaction. Two factor fixed model ANOVA of these remaining data pooled under Replicate revealed significant effects of OP (F_3=69.15, p<0.001) and Time (F_1=50.57, p<0.001). Mean (SE) PC brain ChE activities for DCP, ETP, MMP, and NLD were 3.68 (0.19, n=15), 3.03 (0.24, n=10), 6.50 (0.24, n=10), and 4.35 (0.19, n=15) µmol substrate hydrolyzed/min./g wet mass, respectively, at 24 hours post-dose and were 4.63 (0.19, n=15), 4.32 (0.24, n=10), 6.99 (0.24, n=10), and 5.75 (0.19, n=15) µmol substrate hydrolyzed/min./g wet mass, respectively, at 72 hours post-dose.

Three factor mixed-model ANOVA (Time x Treatment (FO, FD, and FS only) x Replicate) of DCP data revealed only significant interactions while main effects were not significant (Table 3.8). Inspection of the marginal means for the interactions did not suggest a

simple explanation for the observed effects. Variability among replicates seemed similar to variability within replicates. As each of the factors was involved in a significant interaction, the effect of Treatment within each combination of Time and Replicate was examined using one factor fixed-model ANOVA. A significant effect of Treatment was only observed in the comparison at 72 hours post-exposure in Replicate 3 (F_2=5.508, p=0.020) with *post hoc* tests differentiating only between the FD and FS levels of Treatment (Tukey's HSD, p=0.022). Data were then pooled under Replicate to examine effects common among all replicates. The resulting 2 factor fixed-model ANOVA revealed a significant effect of Time while the effect of Treatment approached the significant level (Table 3.8). Mean (SE) brain ChE activity in the FO, FD, and FS treatments were 5.271 (0.201, n=15), 4.988 (0.201, n=15), and 4.957 (0.201, n=15) µmol substrate hydrolyzed/min./ g wet mass, respectively, at 24 hours post-exposure and 5.626 (0.201, n=15), 5.562 (0.201, n=15), and 5.012 (0.201, n=15) µmol substrate hydrolyzed/min./g wet mass, respectively, at 72 hours post-exposure.

Analyses of brain ChE data from simulated field exposures with ETP failed to reveal significant effects of any factor. Pooled mean (SE) ETP brain ChE activity in quail was 7.760 (0.058)(n=90) µmol substrate hydrolyzed/min./g wet mass. These ETP data were normally distributed and pooled brain ChE activity in quail exposed to ETP on the field differed little from that observed in the NC quail.

Three factor mixed-model ANOVA (Time x Treatment (FO, FD, and FS only) x Replicate) of MMP data found no significant effects. Data were pooled under Replicate and the resulting two factor fixed-model ANOVA yielded a significant effect of Time (Table 3.8). Mean

Table 3.8. Results (F statistic, p value) of the analysis of variance (ANOVA) of post-exposure brain cholinesterase data within levels of the factor Organophosphate insecticide, those being dicrotophos (DCP), ethoprop (ETP), methamidophos (MMP), and naled (NLD). Factors within the ANOVA are Treatment (Tr), Time (Ti), and Replicate (Rep). Results that did not approach the significant level ($p>0.10$) are either omitted or not reported (-).

ANOVA Model	Source of Variability	Results (F statistic, p value) from Analysis of Post-exposure Brain Data			
		DCP	ETP	MMP	NLD
3 Factor Mixed	Tr	-	-	-	-
	Ti	-	-	-	13.10, 0.069
	Rep	-	-	-	-
	Tr*Ti	7.23, 0.047	-	-	9.07, 0.033
	Tr*Rep	24.77, 0.004	-	-	-
	Ti*Rep	7.84, 0.041	-	-	5.12, 0.079
2 Factor Fixed	Tr	2.72, 0.072	-	-	8.63, <0.001
	Ti	3.99, 0.049	-	7.46, 0.008	35.35, <0.001
	Tr*Ti	-	-	-	4.78, 0.011

(SE) brain ChE activity in the FO, FD, and FS treatments were 5.482 (0.138, n=15), 5.690 (0.138, n=15) and 5.509 (0.138, n=15) µmol substrate hydrolyzed/min./ g wet mass, respectively, at 24 hours post-exposure and 6.030 (0.138, n=15), 5.904 (0.138, n=15), and 5.669 (0.138, n=15) µmol substrate hydrolyzed/min./g wet mass, respectively, at 72 hours post-exposure.

Three factor mixed-model ANOVA of NLD data revealed a significant interaction in Treatment*Time and a main effect of Time and an interaction of Time*Replicate that both approached the significant level (Table 3.8). Data were pooled under Replicate and the resulting two factor fixed-model ANOVA showed significant effects of Time, Treatment, and the Time*Treatment interaction (Table 3.8). Data were then split into 24 and 72 hour post-exposure groups and one-way ANOVAs performed to investigate Treatment effects in each period. The 24 hour ANOVA revealed a significant effect of Treatment ($F_2=10.21$, $p<0.001$) and *post hoc* tests found the FS treatment to be significantly different than either the FO or FD (Tukey HSD, $p\leq0.007$). A similar analysis of data in the 72 hour post-exposure group failed to find a

significant effect of treatment. Mean (SE) brain ChE activity in the FO, FD, and FS treatments were 5.388 (0.192, n=15), 5.715 (0.192, n=15) and 4.488 (0.192, n=15) µmol substrate hydrolyzed/min./ g wet mass, respectively, at 24 hours post-exposure and 6.480 (0.192, n=15), 5.991 (0.192, n=15), and 5.922 (0.192, n=15) µmol substrate hydrolyzed/min./g wet mass, respectively, at 72 hours post-exposure.

3.3.6 Gut contents

Two factor fixed-model ANOVA (OP x Treatment (FO and FD only)) of gut content wet mass data revealed only a significant effect of the OP (F_3=10.55, p<0.001). *Post hoc* tests on pooled data (FO and FD combined) found the wet mass of gut contents in quail exposed to the ETP spray to be significantly greater than that of quail exposed to other OP sprays (p≤ 0.006)(Figure 3.5). Similar results were obtained with one factor ANOVA and *post hoc* tests of FO and FD data separately except that in *post hoc* tests on the FD data where gut content wet mass of quail exposed to the ETP spray was significantly greater than that of quail exposed either of the MMP or NLD sprays and where results in DCP spray quail were intermediate and not significantly different from any other group. No significant differences in gut content wet masses were found between FO and FD treatments within any OP using independent samples t tests.

The contents of crops and gizzards in quail subject to MMP and NLD sprays in both the FO and FD treatments were all similar. Crops were empty or contained traces of organics and grit. Gizzards contained mostly grit or grit with traces of organics. Quail in the FO treatment exposed to the DCP sprayed field had crops that were empty or contained traces of organics and gizzards that contained clean grit or grit with some organics whereas quail in the FD treatment of

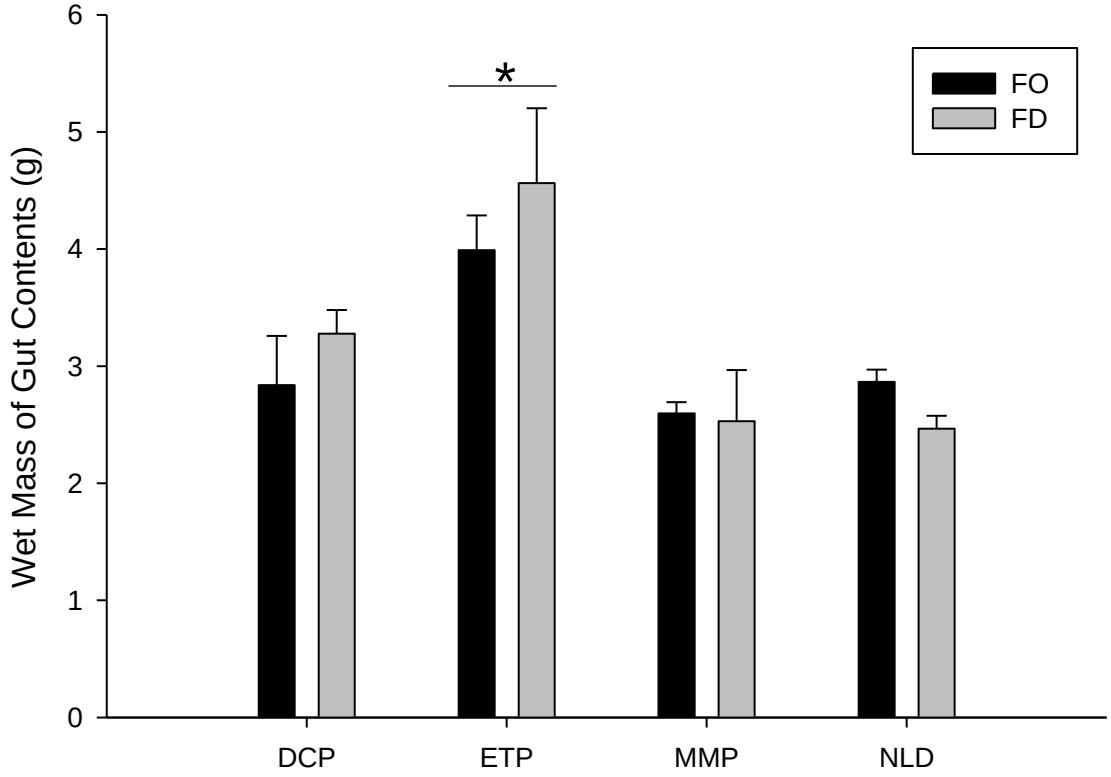


Figure 3.5. Variation in the total wet mass of gut contents (including all material in crop, gizzard, and small intestine) in quail immediately following exposure of quail to two different levels of the factor Treatment (field only (FO) and field plus diet (FD)) on the sprayed simulated field for each level of the factor Organophosphate (OP) insecticide (dicrotophos (DCP), ethoprop (ETP), methamidophos (MMP), and naled (NLD)). Error bars denote (+/-) standard error. The asterisk (*) denotes a significantly higher wet mass of gut contents in ETP-exposed quail (pooled treatments, $p<0.05$).

the same spray had crops that were empty or contained wet feed and gizzards that contained clean grit or grit with feed and/or traces of organics. Quail in the FO treatment exposed to ETP had crops that were empty or contained traces or organics and gizzards that contained grit or grit with a trace to some organics whereas their counterparts in the FD treatment had crops that contained trace to abundant feed and gizzards that contained grit and feed.

A pilot gut contents spray was completed using ETP applied at 2100 g/ha to check that the provision of feed on the simulated field pre-spray would result in a post-spray difference in the wet mass of gut contents in quail. Although this pilot study examined only the contents of the crop and gizzard and excluded the contents of the small intestine, the results were similar to that reported previously for ETP at 1310 g/ha. Crops of quail in the FO treatment were empty while the gizzards contained primarily grit with traces of organics. In contrast, quail in the FD treatments possessed crops that were partly full to full, primarily of the commercial feed used on the field, and gizzards that typically contained this feed and grit. Mean (SE) wet mass of crop and gizzard contents in quail in the FO and FD treatments in this pilot study were 0.884 (0.102) g (n=5) and 1.91 (0.298) g (n=5), respectively. These data failed Levene's test of equal variances and the degrees of freedom in the independent samples t test that followed were adjusted to account for this failure. The difference between the wet mass of crop and gizzard contents in the FO and FD treatments was significant ($t_{4.924}$=3.252, p=0.023).

3.3.7 Exposure Reconstruction

3.3.7.1 Methamidophos

Recovery of ChE activities in plasma and brain from 24 to 72 hours post-exposure in the PC and FO treatments with MMP were plotted as exponential decays to facilitate comparisons and the estimation of effects half-lives (Fig. 3.6). No significant differences between mean plasma ChE activities in the two treatments were found at either post-exposure period. Mean brain ChE activity in the FO treatment was significantly lower than that in the PC treatment at both 24 hours (t_{23}=5.12, p<0.001) and 72 hours (t_{23}=4.52, p<0.001) post-exposure. The estimated

half-lives of ChE inhibition in plasma and brain were 18.2 and 71.8 hours following the PC exposure and 17.7 and 125 hours following the FO exposure, respectively.

3.3.7.2 Dicrotophos

Recovery of ChE activities in plasma and brain from 24 to 72 hours post-exposure in the PC and FO treatments with DCP were plotted as exponential decays (Fig. 3.7). Mean plasma ChE activity was not significantly different between the two treatments at 24 hours post-exposure. However, mean plasma ChE activity in the FO treatment was significantly lower than that in the PC treatment at 72 hours (t_{23}=3.705, p=0.001) post-exposure. Mean brain ChE activity in the FO treatment was significantly higher than that in the PC treatment at both 24 hours ($t_{17.05}$=-6.49, p<0.001) and 72 hours (t_{28}=-5.853, p<0.001) post-exposure. Data in the brain ChE activity comparison at 24 hours post-exposure failed Levene's test of equal variances and the degrees of freedom in the independent samples t test were adjusted to account for this failure. The estimated half-lives of ChE inhibition in plasma and brain were 17.6 and 128 hours following the PC exposure and 41.3 and 222 hours following the FO exposure, respectively.

3.3.7.3 Naled

Recovery of ChE activities in quail tissues following the PC and FO treatments using NLD were again plotted as exponential decays (Fig. 3.8). Mean plasma ChE activity in the FO treatment was significantly lower than that in the PC treatment at 24 hours ($t_{20.63}$=3.559, p=0.002) and but not 72 hours post-exposure. Mean brain ChE activity in the PC treatment was significantly lower than that in the FO treatment at both 24 hours ($t_{21.42}$=-2.85, p=0.009) and 72 hours (t_{28}=-3.52, p=0.001) post-exposure. Data in the plasma and brain ChE activity comparisons

at 24 hours post-exposure failed Levene's test of equal variances and the degrees of freedom in these independent samples t tests were adjusted accordingly. The estimated half-lives of ChE inhibition in plasma and brain were 24.5 and 64.8 hours following the PC exposure and 34.7 and 56.4 hours following the FO exposure, respectively.

3.3.7.4 Ethoprop

Exponential decay plots of recovery of ChE activities in quail tissues following PC and FO exposures to ETP (Fig. 3.9) revealed obvious differences between the treatments. The PC treatment resulted in pronounced inhibition of ChE activity in both plasma and brain at both 24 hours and 72 hours post-exposure whereas the FO treatment produced only a moderate inhibition of ChE activity in plasma at 24 hours post-exposure but almost no response in plasma ChE activity at 72 hours post-exposure and almost no response in brain ChE activity at either time period. No statistical comparisons were made as the effects were evident upon inspection of the data. The estimated half-lives of ChE inhibition in plasma and brain were 21.7 and 106.5 hours following the PC exposure, respectively.

3.4 DISCUSSION

3.4.1 General

Effects following exposures on the simulated field were inconsistent with the assumption upon which screening level avian ERA for pesticide sprays is based, that consumption of contaminated feed is the only route by which birds are exposed. Consumption of contaminated feed on the simulated field was inversely related to toxicity in brain even though oral doses

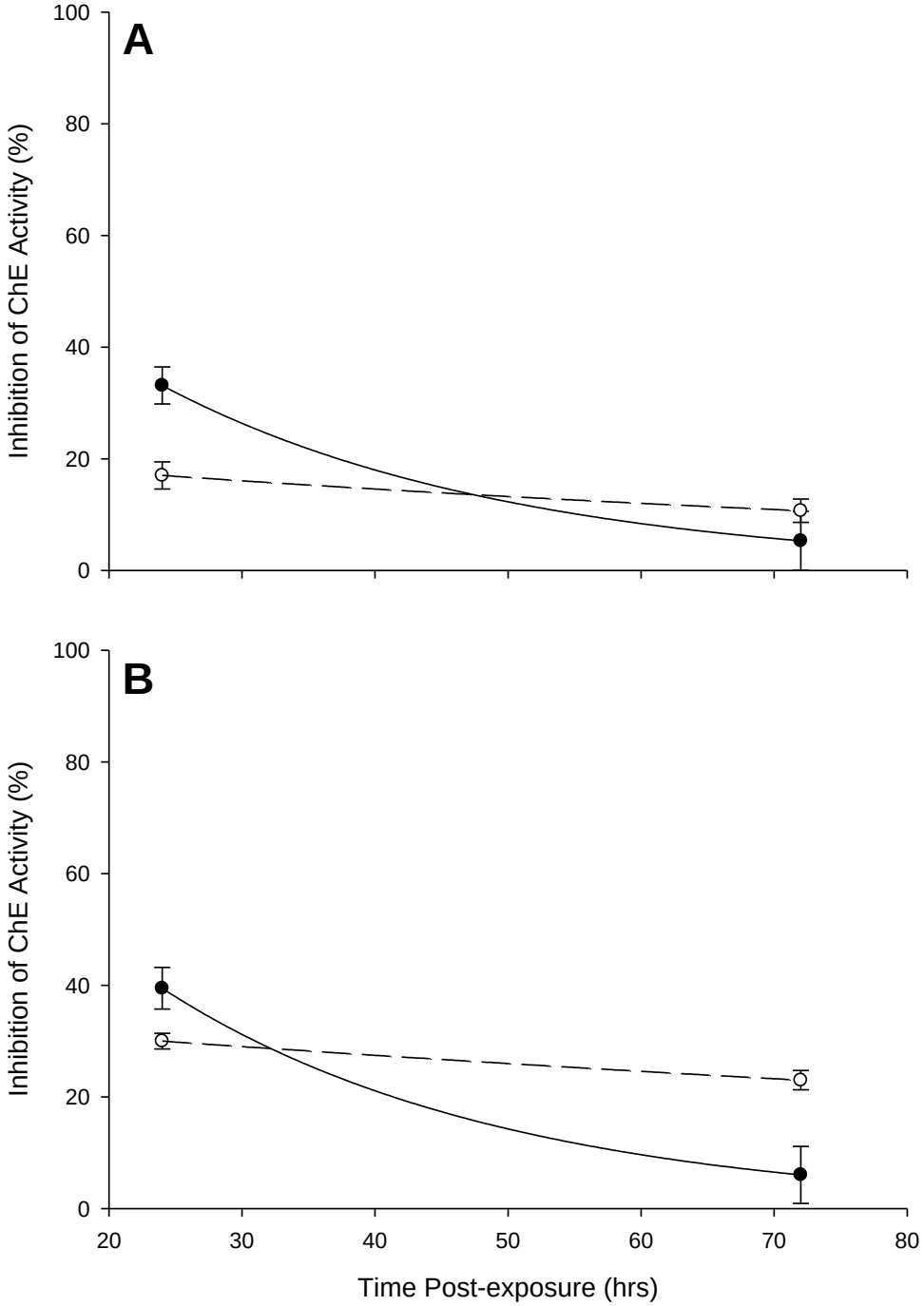


Figure 3.6. Recovery from inhibition of cholinesterase activity in quail plasma (solid line) and brain (dashed line) from 24 to 72 hours post-exposure in two different treatments; **A**) positive control by oral gavage and **B**) exposure to a methamidophos-sprayed simulated field. The recovery curves illustrated are exponential decays. Sample size for each data point is 15 except for positive control brain data where sample size is 10. Error bars denote (+/-) standard error.

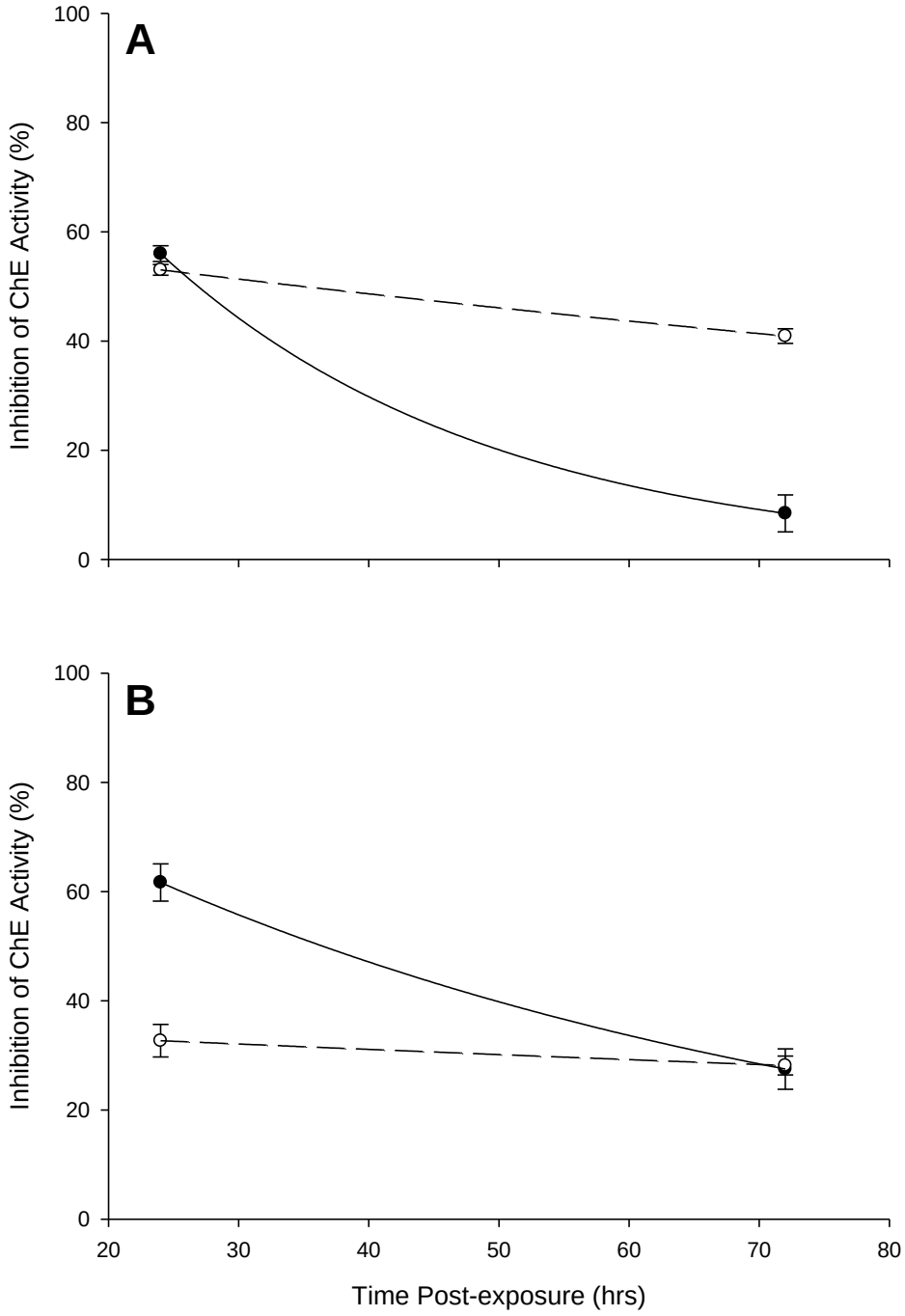


Figure 3.7. Recovery from inhibition of cholinesterase activity in quail plasma (solid line) and brain (dashed line) from 24 to 72 hours post-exposure in two different treatments; **A**) positive control by oral gavage and **B**) exposure to a dicrotophos-sprayed simulated field. The recovery curves illustrated are exponential decays. Sample size for each data point is 15 except for simulated field plasma where sample size is 10. Error bars denote (+/-) standard error.

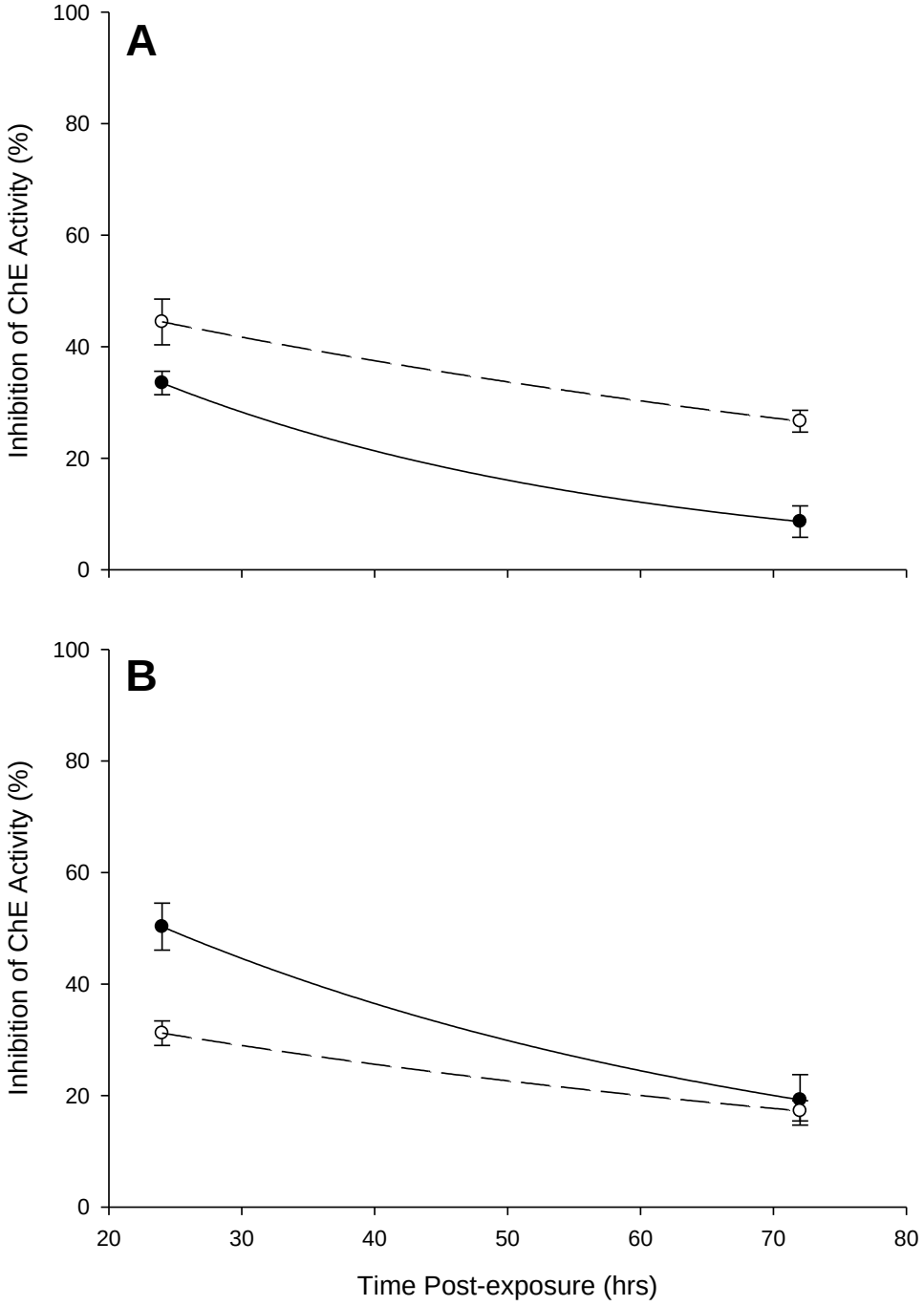


Figure 3.8. Recovery from inhibition of cholinesterase activity in quail plasma (solid line) and brain (dashed line) from 24 to 72 hours post-exposure in two different treatments; **A**) positive control by oral gavage and **B**) exposure to a naled-sprayed simulated field. All the recovery curves illustrated are exponential decays. Sample size for each data point is 15. Error bars denote (+/-) standard error.

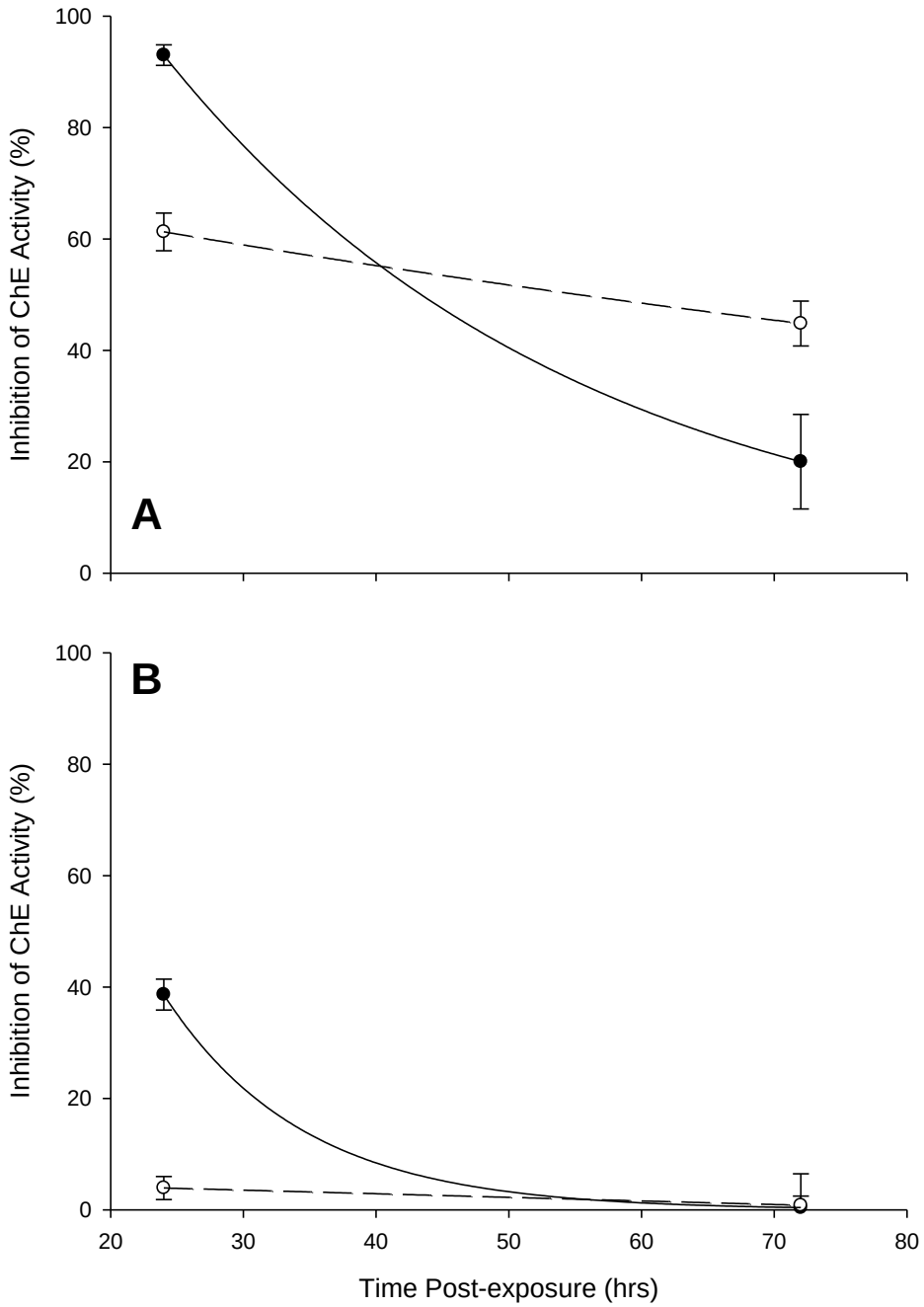


Figure 3.9. Recovery from inhibition of cholinesterase activity in quail plasma (solid line) and brain (dashed line) from 24 to 72 hours post-exposure in two different treatments; **A**) positive control by oral gavage and **B**) exposure to an ethoprop-sprayed field simulated field. All the recovery curves illustrated are exponential decays expect for that in simulated field brain in which is linear. Sample size for data points from the simulated field is 15. Sample size for data points from positive controls is 10 except for plasma at 24 hours post-exposure where sample size is 9. Error bars denote (+/-) standard error.

demonstrated that all four OPs were bioavailable via the oral route. Rather the response of quail to simulated field exposures appeared to be typical of dermal absorption and highly influenced by the solubility of the OP. Recovery from ChE inhibition in avian tissues following simulated field exposures displayed reservoir effects consistent with that known of dermal exposures in mammals and these reservoir effects became more pronounced as log K_{ow} of the active ingredient increased.

3.4.2 Plasma Data and Error Structure

Variation in assay temperature between batches of plasma ChE activity assays caused significant effects in pre-exposure plasma data. While the significant regression of ambient temperature on pre-exposure plasma data indicated that variation in assay temperature was causative, the non-normal distribution of residuals in this regression indicated that sub-optimal laboratory environmental controls were also a source of these effects. As pre-exposure plasma ChE activity assays were generally performed in batches containing either samples from control groups (NC and PC collectively) or samples from treatments on the simulated field (FO, FD, and FS collectively) and proceeded on a replicate by replicate basis within each OP (Appendix B), the significant effects in pre-exposure plasma data were manifest in the factor OP and its interaction with Replicate. Further, the pre-exposure plasma ChE activity in NC and PC groups varied similarly among the OPs and comparisons of effects on the simulated field (i.e. FO vs. FD vs. FS) were relatively free of the confounding influence of assay temperature variation.

Post-exposure plasma ChE activity assays were completed in a manner consistent with pre-exposure plasma samples (Appendix B) and a similar error structure was anticipated. Instead the effects of OP and its interaction with Replicate on post-exposure NC plasma ChE activity

were not significant. Mean ChE activity in post-exposure NC plasma was elevated slightly relative to the grand mean plasma ChE activity calculated from pre-exposure data suggesting that post-exposure plasma ChE assay temperatures were more uniform and typically slightly warmer than those of pre-exposure assays and so less confounded by variation in assay temperature. Further, the influence of between-batch temperature effects and the effects of actual exposures were generally divergent. Significant inhibition of plasma ChE activity at the magnitudes observed simply could not have been the product of between-batch temperature variation. In addition, measures of effect size reveal substantial increases in the effects of the factors Treatment and Time from pre- to post-exposure periods. Inferences made based upon the effects of these factors in post-exposure plasma ChE activity data were not confounded by the effects of between-batch ChE assay temperature variation.

3.4.3 Brain Data and Use of In House Brain Analytical Standard

The avian brain ChE standard proved useful in quantifying between-batch temperature effects on brain ChE assays and the method developed for standardizing activity in the unknowns to median activity in the new analytical standard was found useful in correcting for the effects of assay temperature variation. The multimodal distribution and wide range of activity in measures of activity in the analytical standard suggest that the brain ChE data set for the unknowns would be of questionable utility. Yet the standardized NC brain ChE activity data set passed tests of normality and is almost unmatched in its precision. A limited review of published studies containing measures of reference or control brain ChE activity in birds determined using the assay of Ellman et al. (1961) revealed only one measure of greater precision derived from a reasonable sample size (i.e. $n \geq 10$), that in nonincubating control mallard hens (Fairbrother et al.

1989), while other authors list reference or control brain ChE activity almost as precise (Vyas et al. 2006, Prijono and Leighton 1991, Burn and Leighton 1996). Otherwise, reference or control measures of greater precision were those developed from small sample sizes (i.e. 2 or 3) and so are not truly comparable as small sample sizes result in underestimation of dispersion in a population (Zar 1999). This method also appears to be novel and may prove useful to others, particularly those conducting assays under suboptimal conditions where between-batch temperature effects may be problematic.

3.4.4 Control Groups

3.4.4.1 Negative Control Group

Mean pre-exposure plasma and NC brain ChE activity, 0.734 µmol substrate hydrolyzed/min./mL and 7.83 µmol substrate hydrolyzed/min./g wet mass, respectively, were both consistent with the findings of Soler-Rodriguez et al. (1998) for the same species, 0.838 µmol substrate hydrolyzed/min./mL and 7.32 µmol substrate hydrolyzed/min./g wet mass, respectively.

3.4.4.2 Positive Control Group

Oral dosing demonstrated the ChE-inhibiting properties and oral availability of all four OPs. However, the response in one of the three PC replicates with each of ETP and MMP varied substantially from the responses in the remaining two. This difference was apparent in both tissues with both OPs, being statistically significant in both tissues with ETP and statistically significant in brain with MMP. Data in these replicates were attributed to errors in oral doses within those replicates and excluded from further analyses.

The oral doses of the OPs in the PC treatment were all intended to be one-third the respective LD_{50} in quail. However, the responses varied among OPs. Responses in brain indicated that the oral dose of ETP was high relative to the others while the MMP oral dose appeared relatively low and the oral doses of DCP and NLD appeared intermediate. Regardless of the variation in responses to the oral doses of the OPs, inhibition of ChE activity in plasma and brain and subsequent recovery of ChE activity were typical of a gavage exposure. Recovery from inhibition of ChE activity in plasma was rapid and fairly uniform among the OPs although the extremes of recovery rate in brain varied by a factor of two.

Only the effects following the oral dose of NLD appeared atypical in any way. Inhibition of plasma ChE activity was already lower than that in brain by 24 hours post-dose while recovery of ChE activity in brain was the most rapid among any of the OPs. These effects were interpreted as potentially being a function of extremely rapid recovery of ChE activity in both tissues. Extremely rapid recovery of inhibited ChE activity following an acute exposure to NLD is a phenomenon previously observed in mice (Berteau and Deen 1978). Berteau and Deen (1978) observed rapid recovery of plasma ChE activity in mice from approximately 90% inhibition to approximately 10% induction over a 24 hour period following an acute inhalation exposure to NLD. This recovery of plasma ChE activity was far more rapid than that observed with similar exposures to either of the OPs malathion or chlorpyrifos (Berteau and Deen 1978).

3.4.5 Field Treatments

The addition of feed to the soil surface of the simulated field prior to application of the spray had little impact on the response of quail in the FD treatment. Feed may have been sheltered under the barley canopy during application of the spray and therefore not highly

contaminated and thus of little consequence when it was consumed. In contrast, the FS treatment resulted in the lowest 24 hour post-exposure plasma ChE activity in any of the simulated field treatments with all four OPs, being significantly lower with all except NLD. The presence of birds on the simulated field during application of the spray was a larger contributor to exposure and effects than the presence of feed on the field during application of the spray. The significant increase in inhibition of plasma ChE activity in the FS treatment relative to the other simulated field treatments may have resulted from increases in coverage and concentration in dermal dose associated with exposure to the spray itself. However it is also possible that the spray was inhaled or consumed in preening or entered via the ocular route.

3.4.6 Gut Contents

Inspection and analysis of gut contents from MMP and NLD feeding trials suggest quail in these trials fed little if at all, regardless of which treatment they received. There were also no significant statistical differences in the wet mass of gut contents between either of these two OPs or treatments within them. The measured wet mass of gut contents in feeding trials with these two compounds may, in the majority, simply reflect the sum of a pre-existing burden of grit and secretions. MMP is an irritant (http://www.inchem.org/documents/jmpr/jmpmono/). Hydrolysis of MMP produces methyl mercaptan, another irritant more volatile than MMP itself (Dowling and Seiber 2002). NLD is a fumigant (http://pmep.cce.cornell.edu/profiles/etoxnet) and corrosive to tissues (Berteau and Deen 1978). Quail in MMP and NLD trials may have experienced a degree of discomfort while on the simulated field that deterred them from feeding. In contrast, feed was visible in both the crop and gizzard of quail of the ETP feeding trial and these birds had gut content wet masses significantly higher than quail in feeding trials with other compounds.

Quail on the DCP-sprayed simulated field appeared intermediate with some feeding and others not. A lack of feeding activity on the MMP- and NLD-sprayed simulated field is consistent with findings that the FD treatment had no significant impact on ChE activity in either tissue relative to the response to the FO treatment.

There are two seemingly paradoxical findings that must be explained if one accepts that the alimentary route of exposure is the main contributor to toxicity under field conditions. First, there was an obvious discrepancy in the effects observed in brain when comparing the ETP PC exposures with the ETP simulated field exposures. The ETP gavage resulted in significant inhibition in both plasma and brain while the simulated field exposure resulted in significant inhibition in plasma but no meaningful nor significant inhibition in brain, regardless of treatment. Indeed, the high dose group used in the ETP simulated field pilot study spray had significant inhibition in plasma comparable to that produced by the ETP gavage but only a negligible and not significant inhibition in brain. Quail fed while on the ETP-sprayed simulated field yet effects in brain are lacking. If the route of exposure in both the gavage and simulated field exposures is alimentary, then this discrepancy in effects on brain requires explanation. Second, if quail on the ETP-sprayed field ate too little for effects to develop in brain and they are the group that evidently ate the most while on the simulated field, then how is it that quail exposed on the DCP-, MMP-, and NLD-sprayed simulated field developed effects in brain? It is evident from the feeding trials that quail exposed to MMP and NLD on the simulated field fed little if at all, yet ChE activity in their brain ChE activity was significantly inhibited. Together these two seemingly paradoxical findings imply that a route of exposure other than alimentary was involved in toxicity on the simulated field in these experiments.

3.4.7 Reconstruction of Exposure and Effects

Inhibition and subsequent recovery of ChE activity in quail tissues following exposures on the simulated field varied among the OPs and differed from the patterns observed following oral gavage exposures. Recovery from inhibition of ChE activity following simulated field exposures was explained more parsimoniously by exposure via the dermal route and a dermal reservoir effect that increased with increasing log K_{ow} of the active ingredient.

3.4.7.1 Methamidophos

Recovery of ChE activity in both tissues of MMP-exposed quail was similar between the PC and FO treatments although the FO exposure produced a greater response. With MMP, the most hydrophilic of the OPs, where quail evidently did not feed while on the simulated field, the observed responses are primarily the result of dermal exposure, but the pattern of recovery of ChE activity in both tissues shows no evidence of a reservoir effect.

3.4.7.2 Dicrotophos

The FO treatment produced a lower inhibition of ChE activity in brain but a greater and more persistent inhibition of ChE activity in plasma relative to the PC treatment. Effects in brain indicate a lower dose in the FO treatment while effects in plasma indicate the opposite. The paradox is avoided if dermal exposure is used to explain effects in the FO treatment. The persistent and seemingly greater effects in plasma of the FO treatment relative to the PC treatment are simply the product of a reservoir effect and not the result of a greater dose on the simulated field. DCP was retained in quail skin and then slowly released into circulation after

removal from the simulated field. This slow release of DCP was sufficient to offset some of the recovery of ChE activity in plasma between 24 and 72 hours post-exposure.

3.4.7.3 Naled

Recovery of ChE activity in both tissues following the FO treatment resulted in a typical pattern of ChE inhibition in which inhibition in plasma exceeded inhibition in brain. With DCP, the effects in brain indicate the dose on the simulated field was lower than the gavage dose while results in plasma indicate the opposite. Gradual release of NLD from a dermal reservoir into circulation could have caused the apparent slower rate of recovery of plasma ChE activity in the FO treatment relative to the NLD gavage. The combination of a reservoir effect and extremely rapid recovery combine to explain why such a high application rate was required to achieve the desired 60% inhibition of plasma ChE in pilot study sprays with NLD. Hence, the nearly 60% inhibition of plasma ChE activity produced in the FO treatment relied upon the lag in inhibition of plasma ChE activity associated with the reservoir effect forced by the extremely high application rate.

3.4.7.4 Ethoprop

Inhibition in both tissues following the oral gavage was the greatest observed with any of the OPs. In contrast, the absence of effects in brains of quail in the ETP simulated field treatments, birds which did consume contaminated feed in the FD treatment, is consistent with dermal absorption and storage of a highly lipophilic compound. The rate of diffusion of ETP from the lipophilic regions of quail skin into circulation was very slow such that esterases in quail plasma may have readily scavenged and metabolized ETP entering circulation, thereby

preventing ETP from being distributed to brain. The 6 hour field exposure and relatively low ETP spray application rate were simply insufficient to develop a tissue concentration gradient capable of forcing ETP from the skin reservoir into circulation at a rate high enough to overwhelm the esterases in quail plasma.

3.4.8 Conclusions

This was an exercise in exposure reconstruction. Biomarkers of exposure and effect in quail tissues taken at two different post-exposure intervals revealed differences between the toxicodynamics of two different exposures, the first a gavage which was undeniably of alimentary origin and the second a simulated field exposure in which multiple routes of exposure may have contributed to effects. As the exposures were controlled and replicated experimentally, there was an abundance of supporting exposure data on the oral doses, spray application rates, exposure durations and timing, simulated field conditions, and the experimental OPs. What remained was ignorance regarding how the routes of exposure contributed to toxicity on the simulated field.

Contrasts and similarities in responses in quail among the OPs within the two treatments and between the treatments within each OP proved paradoxical when both treatments were interpreted as resulting from alimentary exposure. Paradoxes were resolved and interpretation simplified when effects on the simulated field were interpreted as resulting primarily from dermal exposure. Dermal reservoir effects varied with log K_{ow} of the OP, being absent with MMP, the most hydrophilic of the OPs used, and so pronounced with ETP, the most lipophilic of the OPs used, that results from pilot study sprays led the author to think that ETP could not cross

the blood-brain barrier in quail (a notion later abandoned when tissues from gavage exposures were analyzed).

Unfortunately, as quail evidently consumed little if any contaminated feed in the FD treatments on the simulated field with MMP and NLD and as feed generally may have been sheltered from spray by the overhanging barley canopy, little may have been learned of how oral and dermal exposures are integrated in the live bird. Regardless, it was evident from these experiments that the response to the 6 hour simulated field exposures resulted primarily from dermal absorption and that the presence of birds on the simulated field during application of the spray was associated with a significant increase in effects beyond that associated with the baseline 6 hour simulated field exposure.

Chapter 4

4.0 SIMULATED FIELD EXPOSURES OF COTURNIX QUAIL TO ORGANOPHOSPHATE INSECTICIDE SPRAYS II: PREDICTIVE EMPIRICAL MODELS FOR ESTIMATING FIELD EXPOSURES

4.1 INTRODUCTION

Environment Canada seeks to improve its methods and practice in assessing the risks of pesticides to birds (Mineau 2007). Specific efforts have been made to develop a risk-based assessment model and incorporate routes of exposure other than through consumption of contaminated feed (Mineau 2007). This model, based largely on prior work (Mineau 2002), seeks to incorporate dermal exposure into the assessment process without having to measure dermal absorption in birds directly (Mineau 2007). Both models (Mineau 2002, 2007) indicate that log K_{ow} of the active ingredient is important in predicting the risks to birds from pesticide sprays and suggest that the more lipophilic, direct acting OPs are delayed in dermal absorption (i.e. slowed by interactions with the lipid-rich SC) and therefore less likely to cause acute toxicity.

The findings of Chapter 3 indicate that effects of the FO treatment on the simulated field are primarily the result of dermal exposure and suggest a reservoir effect is associated with lipids in quail skin. Studies with mammals have revealed significant quadratic polynomial relationships between maximum skin permeation rate, J_{max} (Hinz et al. 1991), or percent of dermal dose absorbed (%Abs) (Yano et al. 1986), and log K_{ow} of the compound under study. The relationships developed by Hinz et al. (1991) and Yano et al. (1986) reflect current thinking that a dermal lipophilic reservoir does exist in mammalian skin and can delay absorption of lipophilic compounds. Here for the FO brain ChE activity inhibition data of Chapter 3 associated with the

nearly equipotent effects in plasma (effects that are irreversible and may result from either slow or rapid dermal absorption), it is assumed that the response in brain is indicative of the extent to which dermal absorption and subsequent distribution exceeded the capacity of plasma esterases to scavenge the OP molecules being distributed from skin to other tissues via the circulatory system. Inhibition of ChE activity in brain resulting from dermal exposure on the simulated field serves as a simple, if somewhat crude, proxy for J_{max} during the exposure.

The objectives of this chapter are to determine the extent to which log K_{ow} of the OPs used in the simulated field experiments described in Chapter 3 predict the responses observed in the FO and FS treatments and to comment on how these findings inform the ecological risk assessment process for pesticide registration.

4.2 Methods

4.2.1 Data

Data generated in the simulated field exposures described in Chapter 3 were used in this curve fitting exercise. Data from the FO treatments with each OP, inferred as resulting primarily from dermal exposure, were used directly in curve fitting. Data from the FD treatments were excluded from the exercise as consumption of contaminated feed was not uniform among the OPs and as effects from consumption of contaminated feed were not expected to be predicted by the solubility of the active ingredient. Estimates of the effect of direct exposure to the OP spray application alone were prepared by assuming the effect of spray would be in addition to the effect of spending six hours on the simulated field. Hence, the effect of spray was estimated by subtracting the effect in the FO treatment from the effect in the FS treatment.

Data points from simulated field exposures used in curve fitting were percent inhibition measured as mean plasma and brain ChE activity in treatment groups (each n=5) relative to the mean pre-exposure plasma ChE activity (n=593) and mean NC brain ChE activity (N=110), respectively. Plasma and brain data points were obtained from simulated field exposures (FO and (FS-FO)) for each combination of OP (DCP, ETP, MMP, and NLD) and Replicate (3 experimental replicates with each OP) used in the simulated field experiments and then fit within each post-exposure period (24 and 72 hours). Twelve data points were generated for each of eight different curve fitting exercises, those being the eight possible combinations of tissue (plasma and brain), post-exposure period (24 and 72 hours), and treatment (FO and (FS-FO)).

4.2.2 Estimates of Solubility for the Active Ingredients

The solubility of the four active ingredients was estimated based upon published octanol-water partition coefficients, as log K_{ow}, for the compounds. These coefficients were obtained from multiple sources including the International Programme on Chemical Safety database (http://www.inchem.org/) and unpublished Environment Canada data provided by a coauthor (Mineau) and then compared. The reported coefficients varied among sources. The octanol-water partition coefficients, as log K_{ow}, used in curve fitting were -0.49, 3.59, -0.80, and 1.38 for DCP, ETP, MMP, and NLD, respectively.

4.2.3 Polynomial Regression

Curve fitting was completed using polynomial regression (Hocking 1996). Linear, quadratic, and cubic curves were fit to the data and then compared. Fit was evaluated based upon the recommendations of Hocking (1996) with consideration being given to the significance of

the relationship, the explanatory ability of the regression equation, the width of confidence intervals, normality in the distribution of residuals, and reasonableness of interpolated predictions. All analyses were run on PASW Statistics 18 (IBM, Chicago, IL, USA).

4.3 Results

4.3.1 Plasma

Polynomial curves could not be fit significantly to the FO and (FS-FO) plasma ChE activity inhibition data sets at either 24 or 72 hours post-exposure. Linear, quadratic, and cubic curves were either not significant, contained residuals that were not normally distributed, or contained interpolated predictions that were nonsensical.

4.3.2 Brain

Quadratic polynomial regressions were significant and found to be the best fit to the FO brain ChE inhibition data sets at both 24 and 72 hours post-exposure (Figure 4.1). The quadratic curve had the most significant fit at 24 hours post-exposure ($F=35.655$, $p<0.001$, $r^2=0.888$). The linear curve had the most significant fit at 72 hours post-exposure but possessed wider confidence intervals than the quadratic curve and residuals that were not normally distributed. This linear fit was rejected in favour of the quadratic curve ($F=30.360$, $p<0.001$, $r^2=0.871$). The resulting quadratic polynomial models fit to the FO brain ChE activity inhibition data sets at 24 and 72 hours post-exposure were Eqs. 4.1 and 4.2, respectively:

$$\text{Inhibition (\%)} = 34.028 + 2.128(\log K_{ow}) - 2.922(\log K_{ow})^2 \quad \text{(Eq. 4.1)}$$

$$\text{Inhibition (\%)} = 23.986 - 2.805(\log K_{ow}) - 1.021(\log K_{ow})^2 \quad \text{(Eq. 4.2).}$$

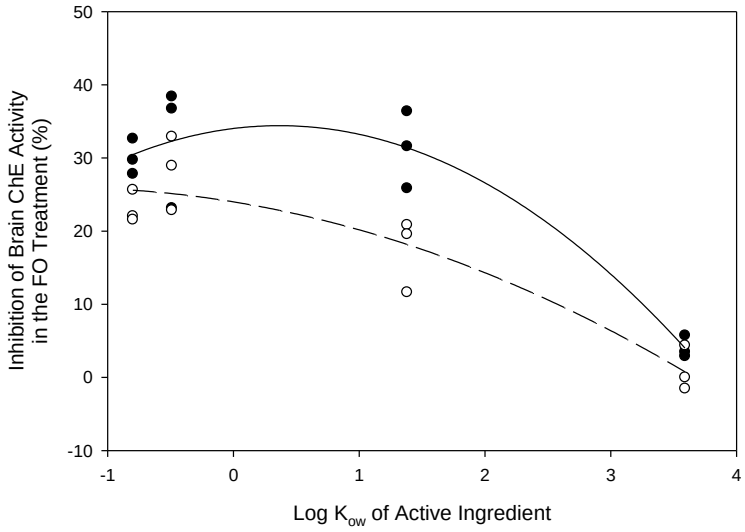


Figure 4.1. Quadratic polynomial curves describing variation in inhibition of brain cholinesterase (ChE) activity in quail with variation of log octanol:water partition coefficient (K_{ow}) of the active ingredient of four different organophosphate insecticide sprays (dicrotophos, log K_{ow}=-0.49; ethoprop, log K_{ow}=3.59; methamidophos, log K_{ow}=-0.80; naled, log K_{ow}=1.38) following six hour simulated field (FO) exposures. Inhibition was determined both at 24 hours post-exposure (closed circles, solid line) and at 72 hours post-exposure (open circles, dashed line). Each data point represents the mean inhibition of a treatment group (n=5) relative to control brain ChE activity (n=110).

A quadratic polynomial regression was found most significant and best fit to the (FS-FO) brain ChE inhibition data set at 24 hours post-exposure ($F=7.360$, $p=0.013$, $r^2=0.621$)(Figure 4.2). No significant fits were obtained for any polynomials for the (FS-FO) data at 72 hours post-exposure. The polynomial model fit to the (FS-FO) brain ChE activity inhibition data set at 24 hours post-exposure was Eqs. 4.3:

$$\text{Inhibition (\%)} = 7.469 + 6.909(\log K_{ow}) - 2.816(\log K_{ow})^2 \qquad \text{(Eq. 4.3).}$$

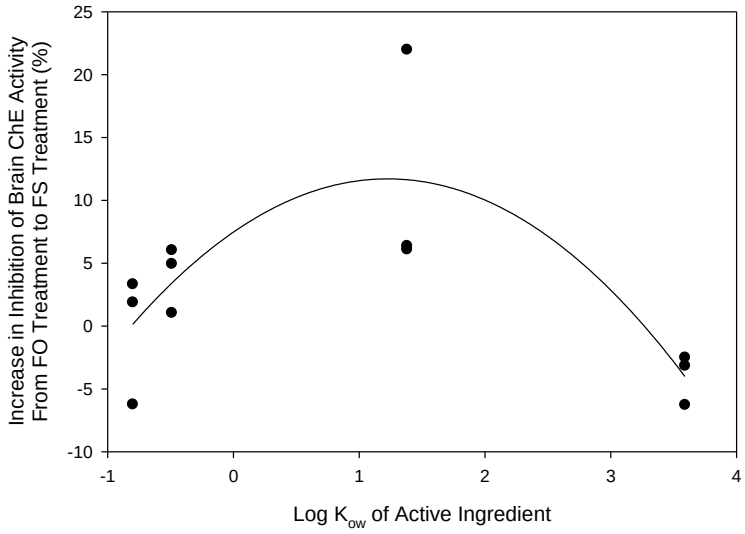


Figure 4.2. Quadratic polynomial curves describing variation in inhibition of brain cholinesterase (ChE) activity in quail with variation of log octanol:water partition coefficient (K_{ow}) of the active ingredient of four different organophosphate insecticide sprays (dicrotophos, log K_{ow}=-0.49; ethoprop, log K_{ow}=3.59; methamidophos, log K_{ow}=-0.80; naled, log K_{ow}=1.38) following direct exposure to the insecticidal spray (FS-FO) exposures. Inhibition was determined at 24 hours post-exposure. Each data point represents the mean inhibition of a treatment group (n=5) relative to control brain ChE activity (n=110).

4.4 Discussion

Log K_{ow} of the active ingredient was significantly predictive of toxicity in brain for these experimental pesticide spray exposures. This was demonstrated for both the 6 hour exposures on the simulated field and for the additional response to the discreet exposure to the spray itself. The quadratic models produced in curve fitting predict that directly acting OPs having log K_{ow} in the range of 0 to 1.5 are those that will be most toxic under field conditions. The findings are also generally consistent with the risk models of Mineau (2002, 2007) in that they indicate that directly acting OPs of balanced lipid:water solubility carry a greater risk to birds relative to more lipophilic ones. The implication is that directly acting OPs with balanced lipid:water solubility

are those most rapidly absorbed and distributed via the dermal route. This appears to be a reasonable conclusion given that it is generally consistent with what is known of mammals (Lane et al. 2012).

It is important to acknowledge the limitations of this data set. Arguably the use of three data points for each OP, one for each experimental spray replicate, represents an instance of pseudoreplication and the data presented here might more cautiously be interpreted as consisting of four data points for each of the three curves developed (Figs. 4.1 and 4.2). Log K_{ow} was the only predictor considered in this effort to avoid an obvious case of overfitting with limited data. Although there is confidence that the observed predictive relationship between inhibition and brain ChE activity and log K_{ow} of the active ingredient has a mechanistic basis consistent with that known in mammals, there is also an acknowledgement that the shape of the curve is poorly characterized due to a lack of data and that it should be confirmed through additional research. In this regard, it is recommended that the relationship between penetration rates through avian skin and the solubility of penetrating compounds be investigated directly. The Organisation for Economic Co-operation and Development (OECD) have established protocols for *in vivo* and *in vitro* skin absorption study methods (OECD 2004a, OECD 2004b, respectively) that have been broadly used with mammals and that presumably could be readily adapted for use with birds. Such efforts could reveal not only the differences (or similarities) in dermal absorption rates between birds and mammals but also the differences (or similarities) in dermal absorption rates between different areas of the bird body (i.e. foot web versus apteria). Arguably this is exactly the type of work Mineau (2007) hopes to avoid.

Although this effort (Chapter 3 and 4, collectively) is constrained by experimental conditions and the use of biomarkers as both surrogates of exposure and toxicity and as proxies

for dermal absorption rates, it suggests that it should be possible to estimate dermal exposures and commensurate risks of directly acting OPs from spray application rates and log K_{ow} and/or other physicochemical properties of the active ingredient. Pilot simulated field study sprays (Chapter 3) revealed that effects in plasma following exposure to the pesticide-sprayed simulated field were linearly related to spray application rate. The quadratic curves developed in this chapter revealed that for a relatively uniform response in plasma, log K_{ow} of the active ingredient predicted effects in brain. These imply that a larger data set covering a range of pesticide concentrations and solubilities can provide estimates of dose and risk. Such a data set is unlikely to be developed based upon field or simulated field studies using live birds due to the time and expense. *In vitro* models, like those of OECD (2004a), seem a more expedient and cost-effective means to compile such data and thereby validate risk-based assessment models.

It should also be noted that the lack of any response associated with consumption of contaminated feed in the FD treatment, either due to avoidance of feed or lack of contamination of feed on the simulated field, made it impossible to gain any insight into how exposures via different routes are integrated into a single response (i.e. ChE inhibition in either plasma or brain). The conventional approach is to assume that responses produced by exposures from different routes are additive, as was utilized by Driver et al. (1991) in estimating toxicity from the dermal route of exposure in their simulated field work. However, this premise remains an assumption that has not been validated and deserves study.

Chapter 5

5.0 COMBINED EFFECT OF SHORT-TERM DEHYDRATION AND SUBLETHAL ACUTE ORAL DICROTOPHOS EXPOSURE CONFOUNDS THE DIAGNOSIS OF ANTICHOLINESTERASE EXPOSURE IN COTURNIX QUAIL (*Coturnix coturnix*) USING PLASMA CHOLINESTERASE ACTIVITY

5.1 INTRODUCTION

Inhibition of cholinesterase (ChE) activity in the brain of wild birds is used to diagnose exposure to anticholinesterase (organophosphorus and carbamate) pesticides (Thompson, 1999). Brain is the preferred tissue for investigating anticholinesterase exposure in bird carcasses because of the preponderance of ChE activity in the organ (Rattner and Fairbrother, 1991) and the persistence of inhibition in the brain (Westlake et al., 1981). Plasma is used to assess exposure in live birds as sampling is non-lethal (Thompson, 1991; Fossi et al., 1992) and as plasma ChE is more sensitive than brain ChE to anticholinesterase exposure (Ludke et al., 1975). The use of plasma also provides for repeated sampling and the ability to perceive changes in activity within a bird over time (Fairbrother et al., 1989; Elliot et al., 2008). However, the utility of plasma in diagnosing exposure is diminished by the high variability in normal plasma ChE activity (Rattner and Fairbrother, 1991; Thompson, 1999).

Several potentially confounding factors must be considered in making a diagnosis of exposure using ChE inhibition (Rattner and Fairbrother, 1991). Within a species, brain ChE activity may vary with age (Grue and Hunter, 1984; Custer and Ohlendorf, 1989) and nutrition (Rattner, 1982) while that in plasma may vary with sex (Ludke et al., 1975; Hill, 1989; Maul and Farris, 2004), age (Ludke et al., 1975; Fairbrother et al., 1990), hormone status (Fairbrother et al., 1989), time of day (Cobos et al., 2010), and season (Hill and Murray, 1987). Studies of the effect of dehydration on ChE activity in birds appear limited to waterfowl possessing salt glands.

The effects of anticholinesterase exposure and an osmoregulatory challenge on ChE activity has been studied in mallards (Herin et al., 1978) and black ducks (Rattner et al., 1983). Salt gland and brain acetylcholinesterase (AChE) activity in mallard ducklings were significantly inhibited by dietary chlorpyrifos exposure while the effect of salt loading in drinking water on these endpoints was not significant (Herin et al., 1978). Similarly, ingestion of fenthion by black ducks caused significant inhibition of both brain and plasma ChE activity while the effect of saline drinking water on these endpoints was not significant (Rattner et al., 1983). Curiously, the combination of fenthion exposure and saline drinking water resulted in plasma ChE inhibition that was significantly different from either fenthion or saline drinking water alone (Rattner et al., 1983). Saline drinking water appeared to dampen the response to fenthion exposure in black ducks (Rattner et al., 1983).

Responses to dehydration have been monitored using measures of plasma osmolality (P_{osm})(Arad et al., 1989) and hematocrit (Hct)(Takei et al., 1988). Increase in P_{osm} was associated with loss of extracellular fluid volume (ECV)(Arad et al., 1989) while increase in Hct was associated with loss of plasma volume (PV)(Takei et al., 1988). No data are available on the effect of hydration state on ChE activity in plasma. However, dehydration is a very common consequence of disease in wildlife. Sick birds may become dehydrated as a result of infection (Joyner et al., 2006), toxicosis (Neimanis et al., 2007; Wynne and Stringfield, 2007) or secondary to reduced mobility and access to water. If dehydration causes a change in ChE activity in the plasma or brain of birds, this effect could confound the diagnosis of anticholinesterase exposure in wild birds submitted to rehabilitation centers, veterinary clinics or diagnostic laboratories.

This study tests the hypothesis that dehydration can cause changes in ChE activity in avian brain and plasma and confound the diagnosis of anticholinesterase pesticide exposure.

5.2 MATERIALS AND METHODS

5.2.1 Experimental Species

Male coturnix quail (*Coturnix coturnix*) between 6 and 26 weeks of age were purchased from Fircrest Farms Ltd. (Langley, BC, Canada).

5.2.2 Animal Care and Housing

The study was conducted in accordance with Protocol #20070071 approved by the Research Ethics Board of the University Committee on Animal Care and Supply at the University of Saskatchewan. Quail were housed communally indoors in a room at the Animal Care Unit in the Western College of Veterinary Medicine, University of Saskatchewan. Quail were provided a commercial, medicated (amprolium 125 mg/kg), crumbled turkey starter feed (Federated Co-operatives Limited, Saskatoon, SK, Canada) and water *ad libidum*. An 8 hours light:16 hours dark cycle was maintained throughout the study. Temperature in the quail room varied from 18 to 22°C during the experiment. Quail deprived of water were isolated within 1.8 meter square cages in the quail room and provided only feed for periods of either 3 or 5 days.

5.2.3 Experimental Designs

Two experimental designs were used in this study, one to assess the response of the quail to different periods of dehydration and another to assess the interaction between dehydration and organophosphorus (OP) pesticide exposure in quail (Figure 5.1). Quail in the dehydration

experiment were grouped by two factors; treatment and replicate. Quail in the combined dehydration-dicrotophos experiment were grouped by three factors; dehydration, OP pesticide exposure, and replicate. The two experimental designs shared several groups of quail. A total of five different combinations of dehydration and OP pesticide exposure in each of three replicates created fifteen groups of quail between the two designs. The sample size in each group was seven and the study required 105 birds. Quail were randomly assigned to different groups. Dependent variables measured included cholinesterase activity in brain and plasma and two measures of hydration state in blood – P_{osm} and Hct.

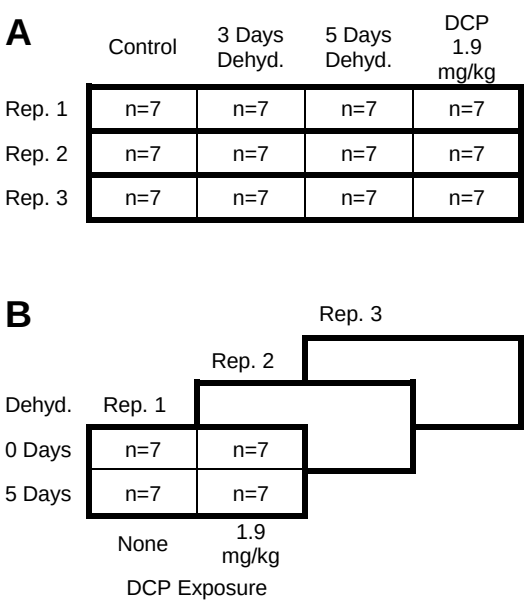


Figure 5.1. The experimental designs for the dehydration experiment (**A**) illustrating grouping by dehydration and replicate and for the combined dehydration-dicrotophos (DCP) experiment (**B**) illustrating grouping by dehydration, DCP exposure and replicate.

5.2.4 Organophosphate Insecticide

Quail were exposed to a dilute (v/v)(dilution factor (DF)=600) aqueous solution of BIDRIN® 8 (dicrotophos 82.0%)(Amvac Chemical Corporation, Los Angeles, CA, USA) by

post-oral gavage at the rate of 1.9 mg a.i./kg, one-third of the estimated LD_{50} for dicrotophos (Environment Canada, unpublished data) provided by a coauthor (Mineau). The gavage was administered 24 hours prior to termination of treatment and collection of tissues such that quail in the 3 and 5 days dehydration treatments had been deprived of water for 2 and 4 days, respectively, when exposed.

5.2.5 Tissue Collection and Storage

Blood samples were obtained before and after the experiment. Approximately 400 µL of blood was collected by jugular veinipuncture using heparinized 1 mL syringes with 27 gauge needles. A portion of the collected blood was used for determination of Hct. Remaining blood samples were centrifuged immediately at 2400xg for 10 minutes at 4°C to separate plasma from red blood cells. Plasma was collected and stored at -80°C until prepared for assay. Quail were euthanized by cervical dislocation following collection of the terminal blood sample. Brains were dissected from carcasses and stored at -80°C until prepared for assay.

5.2.6 Sample Preparation

Plasma samples were diluted (v/v)(DF=12) in 0.5 M phosphate buffer (pH 8) prior to assay. Brain samples were first homogenized in 0.5 M phosphate buffer containing 0.5% Triton X-100 (v/v) while in an ice bath using a Sonic Dismembrator Model 100 tissue homogenizer (Fisher Scientific) for 40 seconds, then further diluted (m/v)(total DF=168) in the same buffer.

5.2.7 Measures of Hydration State in Plasma

Two measures of hydration state were obtained from plasma; 1) P_{osm} and 2) Hct. P_{osm} was measured on a VAPRO® 5520 Vapor Pressure Osmometer (Wescor Inc.). Multiple standards were run on the osmometer at regular intervals to ensure calibration. Hct was determined by centrifugation of whole blood samples in heparinized microcapillary tubes at 10,000xg for 5 minutes. All measures were made in triplicate.

5.2.8 Cholinesterase Assay

Determination of ChE activity in tissues was accomplished using the assay of Ellman et al. (1961) as modified for a microplate reader by Gard and Hooper (1993). Briefly, enzyme activity was measured colorimetrically at 412 nm using acetylthiocholine iodide (AThCh)(>99%, Sigma-Aldrich, UK) as a substrate. Buffer (0.5 M phosphate, pH=8), a solution of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB)(Sigma-Aldrich, USA) in buffer (0.5 M phosphate, pH=7) (final concentration (FC)=$3.23x10^{-4}$ M), sample preparation, and substrate in buffer (0.5 M phosphate, pH=8)(FC=$1x10^{-3}$ M) were added to 96 well microtiter plates (Nalge Nunc International)(total volume=260 µL). Buffer was maintained at 25°C while reagent solutions and samples were maintained in ice baths. Substrate was added lastly and immediately prior to assay to initiate the reaction. ChE assay results were read on a SpectraMax® 190 microplate reader (Molecular Devices Corporation, Sunnyvale, CA, USA) set in absorbance mode and at 25°C. An analytical acetylcholinesterase standard was prepared from the membranes of equine red blood cells in general accordance with the method developed for human red blood cells by Dodge et al. (1963) as modified by Hansen and Wilson (1999). The standard was run on all microplates assayed. All measures were made in triplicate.

5.2.9 Statistics

Hypotheses were tested using analysis of variance (ANOVA)(Zar, 1999). Both dehydration and OP exposure were treated as fixed-effects factors while replicate was treated as a random-effects factor and fixed- or mixed-model ANOVA was employed, as appropriate. *Post hoc* means tests were completed using Tukey's HSD method. Linear regression was employed to investigate correlations between plasma ChE activity and other plasma parameters. Significance was associated with $p<0.05$. All analyses were run on PASW Statistics 18 (IBM, Chicago, IL, USA).

5.3 RESULTS

5.3.1 Sample Losses

One quail in the control treatment group did not yield sufficient blood at the end of the protocol for determination of post-exposure P_{osm} and Hct. Plasma ChE activity was the sole blood parameter determined for that bird. Another quail in the combined dehydration-dicrotophos exposure died in housing and post-exposure samples were not obtained.

5.3.2 Control Brain Cholinesterase Activity and Pre-exposure Plasma Parameters

Mean (coefficient of variation (CV)) brain ChE activity in control quail was 6.99 (16%) µmol substrate hydrolyzed/min./g wet mass (n=21). Pre-exposure values for P_{osm}, Hct, and plasma ChE activity were measured in the experimental and other quail kept in housing between 1 and 2 weeks prior to the start of the experiment to establish reference values for these parameters (Table 5.1). The coefficients of variation for the plasma parameters revealed a

ranking of normal homeostatic ranges from narrowest to broadest being P_{osm}<Hct<plasma ChE activity.

Table 5.1. Mean, coefficient of variation (CV), and sample size (n) for plasma osmolality (P_{osm}), hematocrit (Hct), and plasma cholinesterase (ChE) activity in experimental and extra quail prior to experimentation.

Parameter	P_{osm}	Hct	Plasma ChE Activity
Mean	298.7 mosmol/kg	42.7 %	0.765 µmol substrate hydrolyzed/min./mL
CV	2.5 %	7.5 %	21 %
n	111	111	111

Measures and variability of cholinesterase activities in tissues of our quail were consistent with that observed in this species by Soler-Rodriguez et al. (1998) who reported mean (CV) brain and plasma ChE activities of 7.32 (11%) µmol substrate hydrolyzed/min./g wet mass and 0.838 (32%) µmol substrate hydrolyzed/min./mL, respectively.

5.3.3 Effects on Brain ChE Activity

No replicate effect was observed in either of the dehydration or combined dehydration-dicrotophos experiments and data from replicates were pooled within each experiment. Treatment had a significant effect on brain ChE activity in the dehydration experiment (F_3=55.28, p<0.001). *Post hoc* tests revealed that brain ChE activity in dicrotophos-exposed quail was significantly lower than the activity in other treatments (Figure 5.2). Similarly, dicrotophos exposure had a significant effect on brain ChE activity in the combined dehydration-OP exposure experiment (F_1=211.79, p<0.001)(Figure 5.3) while dehydration and the dehydration*dicrotophos interaction were not significant.

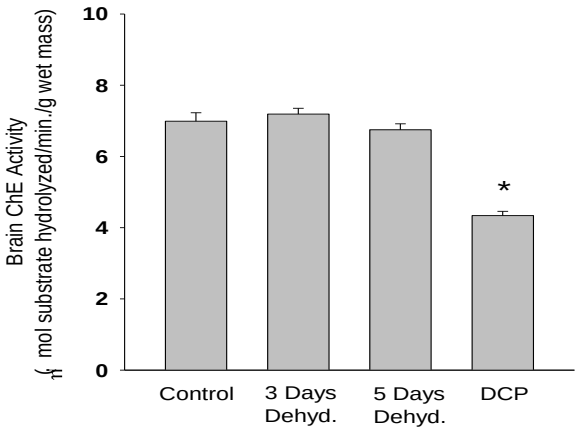


Figure 5.2. Variation in brain cholinesterase activity in quail within treatments: control (n=21); 3 days dehydration (n=21); 5 days dehydration (n=21); and dicrotophos (DCP)(n=21). Error bars denote (+/-) standard error. The asterisk (*) denotes a significantly lower brain cholinesterase activity in DCP-exposed quail than in quail in other treatments ($p<0.05$).

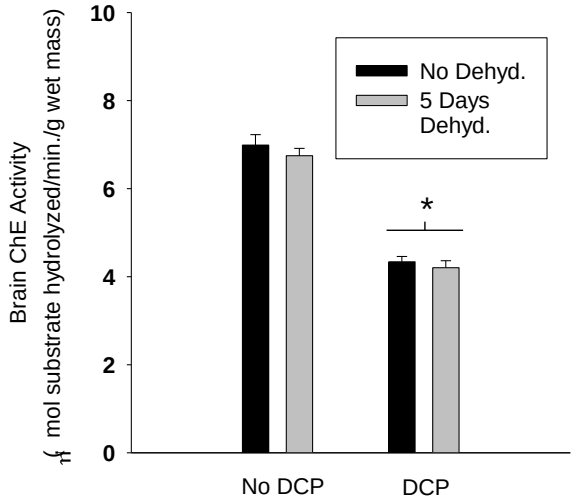


Figure 5.3. Variation in brain cholinesterase activity in quail within different combined treatments of dicrotophos (DCP) exposure and dehydration: no DCP, no dehydration (n=21); no DCP, 5 days dehydration (n=21); DCP (1.9 mg/kg), no dehydration (n=21); and DCP (1.9 mg/kg), 5 days dehydration (n=20). Error bars denote (+/-) standard error. The asterisk (*) denotes a significantly lower brain cholinesterase activity in DCP-exposed quail than in quail not receiving DCP ($p<0.05$).

5.3.4 Effects on Plasma Parameters

The replicate effect on P_{osm} and Hct was not significant in either of the experiments and data in these experiments were pooled. Treatment had a significant effect on both P_{osm} (F_3=83.02, p<0.001) and Hct (F_3=14.38, p<0.001) in the dehydration experiment. *Post hoc* tests showed significant increases in P_{osm} (Figure 5.4A) and Hct (Figure 5.4B) with increases in the period of dehydration. Dehydration had a significant effect on P_{osm} (F_1=212.87, p<0.001) in the combined dehydration-dicrotophos experiment while the other effects were not significant. Both dehydration and dicrotophos exposure had significant effects on Hct (F_1=31.88, p<0.001 and F_1=5.96, p=0.017, respectively) in the combined dehydration-dicrotophos experiment while the interaction was not significant.

The replicate effect on plasma ChE activity was not significant in either experiment although analysis of both experiments did reveal significant effects of the final interaction terms (dehydration experiment, treatment*replicate, $F_{2,6}$=3.85, p=0.002; combined dehydration-dicrotophos experiment, dehydration*dicrotophos*replicate, $F_{2,71}$=6.50, p=0.003). These interactions were thought to result from experimental error**.** A comparison of the ANOVAs for each replicate and pooled data in the interaction experiment (Table 5.2) revealed significant dehydration*dicrotophos interactions in Replicates 1 and 2 and an effect of dicrotophos that is not significant in Replicate 2, none of which influence the results using pooled data. This is consistent with the effects of random experimental errors in the replicates that weaken when data are pooled. The failure of ANOVA to reveal a significant effect of dicrotophos in Replicate 2 can be attributed to anomalously low plasma ChE activity in the 5 days dehydration group as a focused independent samples t test comparing control and

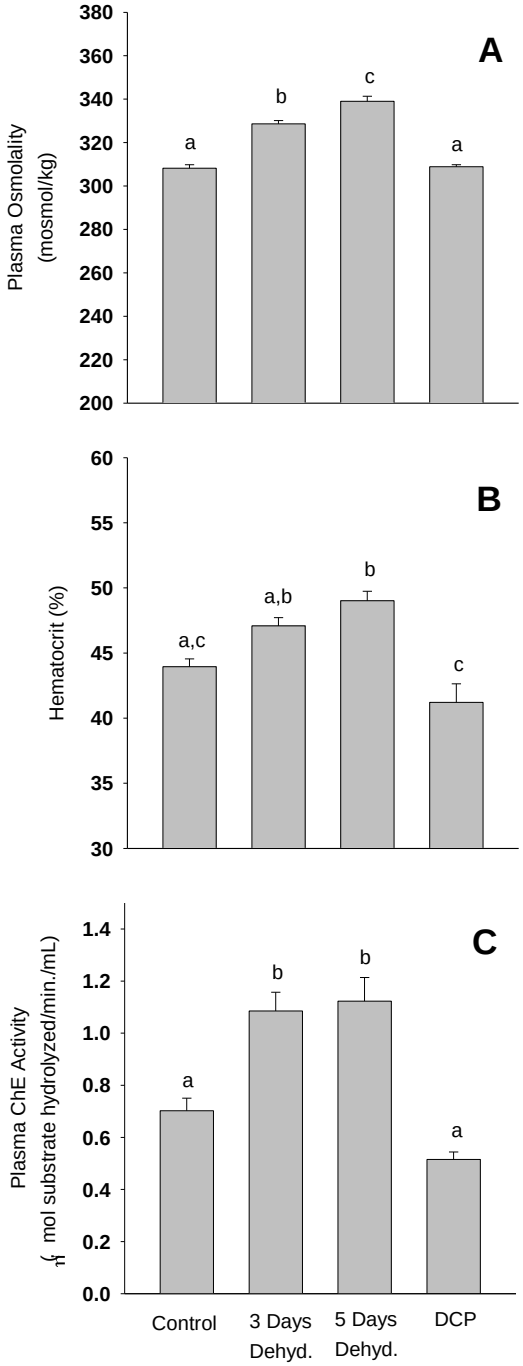


Figure 5.4. Variation in plasma osmolality (**A**), hematocrit (**B**), and plasma cholinesterase (ChE) activity (**C**) within treatments: control (n=20 for **A** and **B**, n=21 for **C**); 3 days dehydration (n=21); 5 days dehydration (n=21); and dicrotophos (DCP)(n=21). Error bars denote (+/-) standard error. Lower case letters denote homogenous subsets within each data set ($p<0.05$).

Table 5.2. Significance of main and interaction effects on plasma ChE activity within the interaction experiment for each replicate and the pooled data.

Data Source	Replicate 1	Replicate 2	Replicate 3	Pooled Data
Dehydration	<0.001	0.001	0.001	<0.001
Dicrotophos	<0.001	0.883	0.003	<0.001
Interaction	0.013	0.045	0.342	0.427

dicrotophos-exposed groups does reveal the effect (t=3.31, p=0.002). Analysis of pooled data in the dehydration experiment revealed a highly significant effect of treatment (F_3=21.33, p<0.001) and *post hoc* tests showed significant increases in plasma ChE activity with dehydration (Figure 5.4C). A similar analysis in the combined dehydration-dicrotophos experiment revealed highly significant and opposing effects of dehydration (F_1=39.32, p<0.001) and dicrotophos (F_1=15.22, p<0.001) while the interaction was not significant.

Ordinary least-squares (OLS) regression using measures of within-bird change in P_{osm} and within-bird change in plasma ChE activity from 69 quail in the control and dehydrated treatment groups revealed a significant positive correlation between the parameters (F_1=26.52, p<0.001)(Figure 5.5A). Observations in the 5 days dehydration group of Replicate 2 (Figure 5.5A, open circles) plotted below the regression line suggesting that the increase in plasma ChE activity in this group was lower than should be expected, arguably the result of experimental error. Further, variation in the within-bird change in P_{osm} accounted for approximately 28% of the variation in within-bird change in plasma ChE activity. The regression slope was 0.00910 (p<0.001). The regression intercept was -0.010 and not significant. Regression through the origin (RTO) (Eisenhauer 2003) was performed to assess whether the origin was a better fit to the data than the intercept of the OLS regression line. The standard errors of the intercepts for the OLS and RTO lines were 0.234 and 0.232, respectively, suggesting that the origin was indeed a better

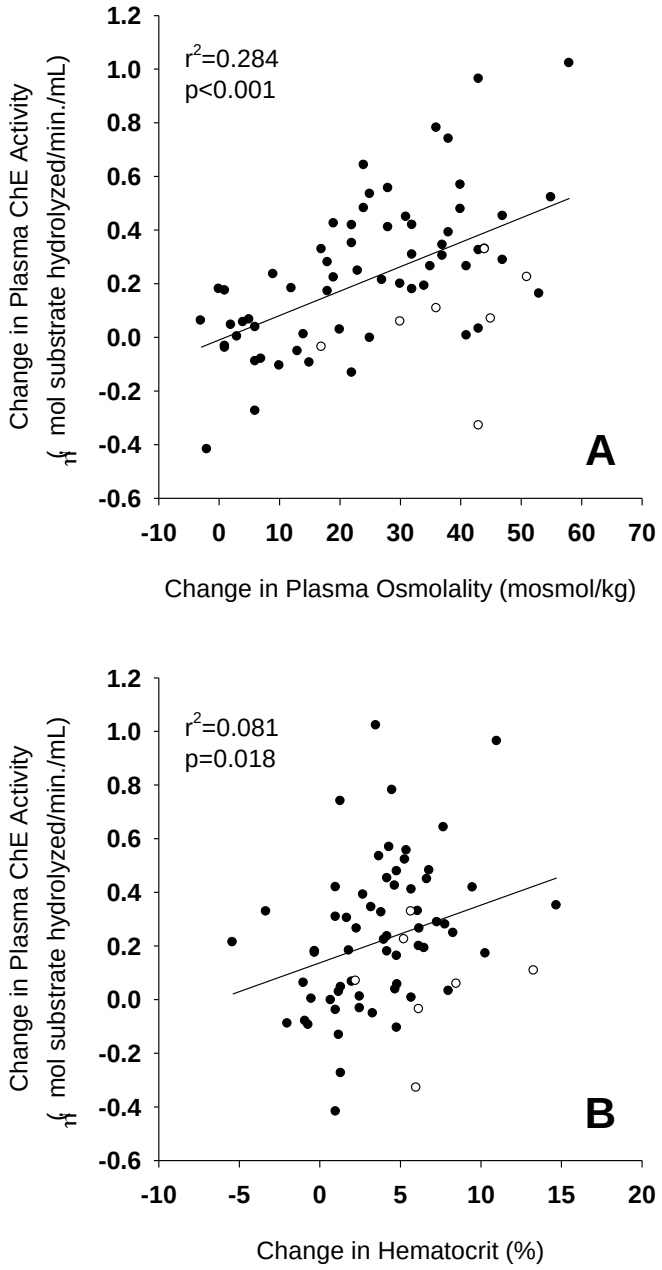


Figure 5.5. Linear regressions between plasma parameters in quail not exposed to dicrotophos using change in plasma osmolality from pre- to post-exposure and change in plasma cholinesterase activity from pre- to post-exposure (**A**)(n=69); and change in hematocrit from pre- to post-exposure and change in plasma cholinesterase activity from pre- to post-exposure (**B**)(n=69). Open circles denote observations in the 5 days dehydration group of Replicate 2 while filled circles denote all other observations.

fit to the data than the OLS line intercept. Changes in P_{osm} and plasma ChE activity apparently commenced simultaneously in response to dehydration. A similar regression using measures of within-bird change in Hct and within-bird change in plasma ChE activity from the same 69 quail revealed a significant positive correlation between these parameters (F_1=5.89, p=0.018) (Figure 5.5B). However, within-bird change in Hct accounted for only 8.1% of the variation in within-bird plasma ChE activity. The regression slope was 0.0215 (p=0.018). The intercept was 0.137 (p=0.006). This intercept was both significant and greater than zero, suggesting that increases in both P_{osm} and plasma ChE activity preceded the increase in Hct in response to dehydration.

The potential for the combined effects of dehydration and dicrotophos to confound the diagnosis of anticholinesterase exposure using plasma ChE activity was investigated by pooling the data from all replicates and comparing the resulting data set with the reference pre-exposure plasma ChE activity using one factor ANOVA. The ANOVA revealed a significant effect of treatment (F_4=22.81, p<0.001). *Post hoc* tests were performed to compare treatments (Figure 5.6A). Both the dehydration and dicrotophos treatment groups were significantly different from all others while there was no significant difference between the reference pre-exposure, control and combined dehydration-dicrotophos exposure groups.

Plasma ChE activities in the four treatment groups were then adjusted using the OLS regression line between within-bird change in P_{osm} and within-bird change in plasma ChE activity (Figure 5.5A). Adjusted plasma ChE activities (ChE_{adj}) were calculated from observed plasma ChE activities (ChE_{obs}) and the within-bird change in P_{osm} (ΔP_{osm}) using Eq. 5.1:

$$ChE_{adj} = ChE_{obs} - 0.009(\Delta P_{osm}) \quad \text{(Eq. 5.1)}$$

where the coefficient, 0.009, is the slope of the regression line. The ANOVA was then rerun using the adjusted plasma ChE activities for the treatment groups and the original plasma ChE

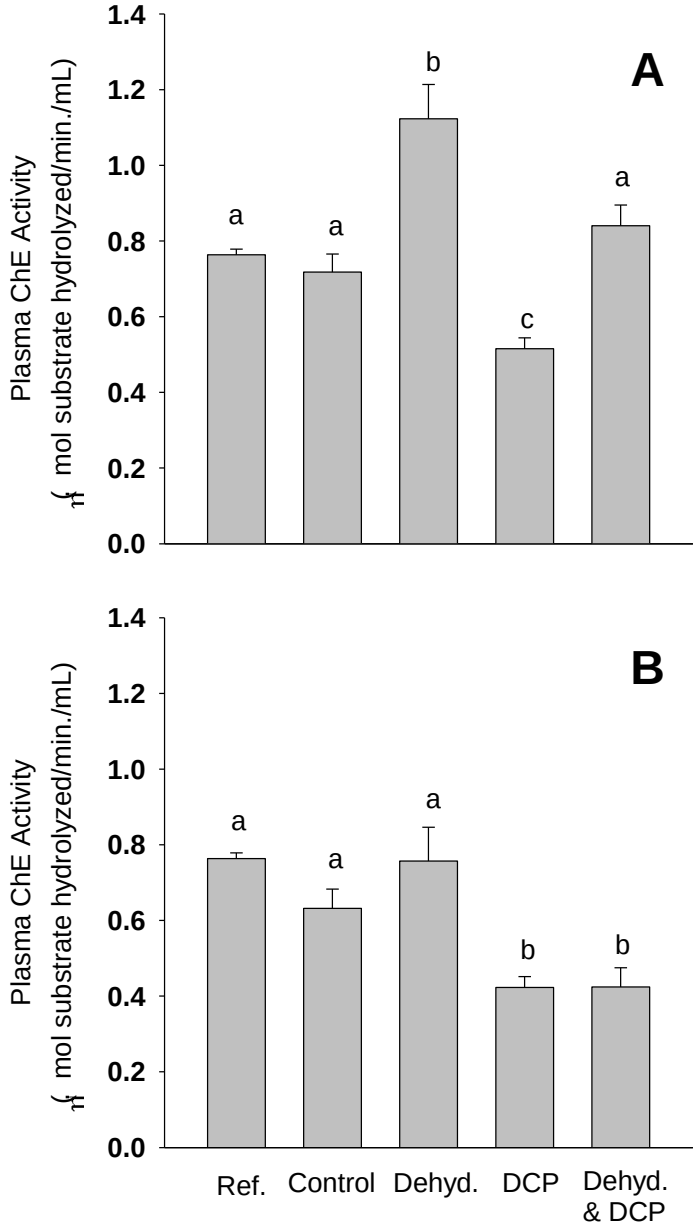


Fig. 5.6. Plasma total ChE activity in quail by treatment group using (**A**) original data and (**B**) data adjusted using change in plasma osmolality from pre- to post-exposure where treatment groups are: **1**) reference (pre-exposure) quail (n=111), **2**) control quail (n=20), **3**) dehydrated (5 days) quail (n=21), **4**) dicrotophos (DCP) exposed quail (n=21), and **5**) dehydrated (5 days) and DCP exposed quail (n=20). Lower case letters denote homogenous subsets within each data set ($p<0.05$). Error bars denote (+/-) standard error.

activities from the reference pre-exposure observations. A significant effect of treatment was revealed (F_4=20.47, $p<0.001$) and a more robust portrayal of dicrotophos exposure emerged in *post hoc* tests (Figure 5.6B). Two homogenous subsets were found, each associated by their exposure (or lack of) to dicrotophos.

5.4 DISCUSSION

The induction of dehydration in quail using water deprivation resulted in significant effects in all dependent variables measured except brain ChE activity. Dehydration neither affected the ability of dicrotophos to inhibit ChE activity in brain nor altered ChE concentrations in that tissue by causing a change in brain wet mass. This may reflect preferential maintenance of the hydration state in the brains of quail at the expense of water loss from other body compartments during dehydration, a response known in mammals (Nose et al., 1983).

The observed increases in P_{osm} and Hct are likely indicative of decreased extracellular fluid volume (ECV) and plasma volume (PV) during water deprivation, respectively. Increases in these plasma parameters have been observed in birds in response to water deprivation, although not always concurrently. Significant increases in P_{osm} have resulted from short term water deprivation in chickens (Arad et al., 1985), pigeons (Arad et al., 1989; Carmi et al., 1994), starlings (Roberts and Dantzler, 1989) and Japanese quail (Takei et al., 1988). However, among these studies, only the work with Japanese quail found a significant increase in hematocrit associated with water deprivation (Takei et al., 1988). Arad et al. (1985) examined the response of chickens subjected to 48 hours of water deprivation and observed significant increases in P_{osm}. However, their work focused on the relation between osmoregulation and hormones and no inferences were made regarding ECV and PV (Arad et al., 1985). Arad et al. (1989) later

conducted similar work with pigeons and measured both P_{osm} and Hct. Forty-eight hours of water deprivation caused a significant increase in P_{osm} but little variation in Hct, from which they inferred that PV had been maintained at the expense of water loss from other compartments of the ECV (Arad et al., 1989). The authors also noted that their test pigeons had been conditioned to heat stress and mentioned contrasting findings in their unpublished work with pigeons not acclimated to heat, wherein PV was lost during water deprivation (Arad et al., 1989). Carmi et al. (1994) subjected pigeons to water deprivation under different temperature regimes and observed a significant increase in P_{osm} and non-significant variation in Hct in all treatments. Both total body water (TBW) and PV were measured directly and it was found that the pigeons were capable of maintaining PV but not TBW during water deprivation except under the highest ambient temperature tested (40°C), wherein PV was reduced significantly (Carmi et al., 1994). Concurrent significant increases in P_{osm} and non-significant changes in Hct were also observed (Carmi et al., 1994). The findings of Roberts and Dantzler (1989) with starlings were similar. Starlings subjected to 24 hours of water deprivation had significantly lower body weight and significantly increased P_{osm} while Hct varied little and PV was maintained (Roberts and Dantzler, 1989). In contrast, Takei et al. (1988) deprived Japanese quail of water and measured body weight, blood volume, P_{osm} and Hct. Increases in both P_{osm} and Hct were observed shortly after water deprivation commenced and became significant after only 10 hours (Takei et al., 1988). Concurrent decreases in body weight and blood volume were also observed and became significant after only 4 and 7 hours, respectively (Takei et al., 1988). Takei et al. (1988) concluded that their quail were not capable of maintaining blood volume during water deprivation. This observation is noteworthy given the close taxonomic relation between Japanese quail and the species we used, as our findings suggest a similar phenomenon in Coturnix quail.

This work was not intended as an investigation of osmoregulatory function in quail and so we lack measures of body weight, TBW, ECV and PV from which we could make inferences regarding the movement of water between various body compartments. We instead rely on our measures of P_{osm} and Hct as proxies for change in ECV and PV, respectively. Quail in our experiments had significant increases in P_{osm} after both 3 and 5 days of water deprivation and in Hct after 5 days of water deprivation which we associate with loss of ECV and PV, respectively. The linear regression relationships used to explore correlations between P_{osm} and plasma ChE activity and between Hct and plasma ChE activity then inform us as to the timing of these changes and the distribution of plasma ChE activity within body water compartments. The regression constants indicate that changes in P_{osm} and plasma ChE activity were concurrent and preceded change in Hct. This suggests that plasma ChE occupies a larger pool of body fluid than just the PV. Further, the regression slopes indicate that changes in P_{osm}, plasma ChE activity and Hct were not proportional. A 1% change in P_{osm} was associated with a 3.5% change in plasma ChE activity which, in turn, was associated with a 3.0% change in Hct. This also suggests differences among the volumes of the body water compartments associated with each of P_{osm}, plasma ChE activity and Hct. P_{osm} variation is associated with change in the concentration of electrolytes which are distributed throughout the ECV. Hct is a measure unique to PV. It appears that plasma ChE activity is therefore not limited to plasma but instead occupies a larger pool of body fluid intermediate between PV and ECV and we suggest lymph as a repository for ChE activity. Observations in mammals suggest that ChE in plasma and thoracic duct lymph are coupled (Lindena et al., 1986). Lindena et al. (1986) measured the thoracic duct lymph to plasma ratio of ChE activity in dogs, rabbits, rats and mice and inferred a concentration gradient forcing the transport of ChE from plasma to lymph. If these observations in mammals are true for our

quail, then we have a mechanism that explains our observations. Dehydration initially caused a reduction in the ECV while PV was maintained. P_{osm} and plasma ChE activity both increased but the proportional increase in P_{osm} was less than that of plasma ChE activity as plasma ChE activity is confined to a smaller subset of the ECV than P_{osm}. Eventually, as dehydration proceeded, the ability of the quail to maintain PV was exceeded and loss of PV resulted. Then Hct increased with both P_{osm} and plasma ChE activity. Here the proportional increase in Hct was smaller than that in plasma ChE activity, suggesting the quail continued to favor conservation of PV over other compartments of the ECV as dehydration progressed.

The combination of 5 days dehydration and sublethal acute oral dicrotophos exposure at one-third the estimated oral LD_{50} of dicrotophos in quail resulted in near normal plasma ChE activity. Dehydration confounded the diagnosis of dicrotophos exposure in quail using plasma ChE activity. This finding is similar in trend with that observed by Rattner et al. (1983) in which an osmoregulatory challenge associated with saline drinking water dampened the plasma ChE inhibition response of black ducks to fenthion exposure. It is also apparent that if a correlation between changes in P_{osm} and plasma ChE activity can be established, then it may be possible to adjust plasma ChE activities for dehydration effects.

These findings are of importance for two reasons. Firstly, the effect of dehydration on plasma ChE activity in this species is antagonistic to the effect of dicrotophos. Clinicians and investigators using plasma ChE activity to diagnose or quantify exposure to the anticholinesterase pesticides should note that dehydration can mask the effects of anticholinesterase agents on plasma ChE activity. Secondly, the observed relationships between changes in P_{osm} and Hct with change in plasma ChE activity reveal that ChE of plasma actually occupies a larger pool of body fluid. This suggests that the mechanisms involved in

osmoregulation contribute to some of the natural variability of plasma ChE activity. We note that much effort has been put into characterizing the variation of plasma ChE activity with season, time of day, cohort, hormone status, etc. while little effort, if any, has been put into describing the mechanisms underlying this natural variation. Here we postulate that the loss of plasma ChE activity through daytime hours known in some bird species, as observed in clay-colored robin (*Turdus grayi*) by Cobos et al. (2010), for example, may simply reflect a net gain in body water with daytime feeding and drinking. In that regard, we suggest that study of the mechanisms responsible for natural variation in plasma ChE activity is warranted and recommend osmoregulation for further investigation.

Chapter 6

6.0 TELEMETRY OBSERVATIONS OF HYPOTHERMIA AND TACHYCARDIA IN COTURNIX QUAIL IN RESPONSE TO SUBLETHAL ORAL DOSES OF ORGANOPHOSPHATE PESTICIDES AND COLD-STRESS

6.1 INTRODUCTION

Heinz (1989) asked if the sublethal effects of environmental pollutants are truly harmful to bird populations and noted two principal failings in the science of the day in answering the question. Firstly, there was little evidence that the sublethal effects of contaminants observed in the lab actually occurred in the field (Heinz 1989). Secondly, proof was required that a sublethal effect observed in the lab would cause mortality in the field (Heinz 1989). Two years later, the review of Grue et al. (1991) summarized many of the sublethal effects associated with exposure to cholinesterase (ChE) inhibitors, the organophosphorus (OP) and carbamate pesticides. In that same year, Mineau (1991) commented upon some of the sublethal effects of ChE inhibitors that may influence the long term survival of exposed wildlife. Grue et al. (1997) revisited the topic in 1997 and discussed at length the sublethal effects they thought most likely to adversely impact wildlife populations within regions where ChE inhibitors are used. Each noted either that the data of the day either provided only for generalizations about the effects likely to result from given levels of exposure (Grue et al. 1991, Mineau 1991) or were of limited utility to regulators (Grue et al. 1997). Regrettably, it appears that there has been little progress in the many years since these publications.

Outward clinical signs in birds of sublethal cholinergic toxicity include ataxia, blindness, convulsions, dyspnea, hyperexcitability, lethargy, piloerection, slurred vocalizations, and tremors, among others (Grue et al. 1991). Sublethal behavioural effects include altered activity patterns, reduced food and water intake, aggression, and adverse effects on nest defense and

parental care (Grue et al. 1991, Grue et al. 1997). These behavioral deficits may impair survival or reproduction (Grue et al. 1991, Grue et al. 1997). Adverse effects on thermoregulation (Grue et al. 1991, Grue et al. 1997) and cardiac function (Grue et al. 1991) have also been observed. It is these latter effects on thermoregulation and cardiac function that were the focus of this study.

The use of radio telemetry implants to monitor physiological responses to OP pesticides is a better developed practice in mammals (Gordon 1996, Gordon and Padnos 2000, Smith and Gordon 2005) than it is with birds. Telemetry has been used to investigate OP-induced changes in cardiovascular function or thermoregulation in mammals (Gordon and Padnos 2000, Smith and Gordon 2005), but we are not aware of any similar application in birds. McFarland and Lacy (1968) investigated parathion's effects on heart rate in quail and ducks using restrained birds hard-wired to an Offner Dynograph. Underwood (1994) later used telemetry to investigate the diurnal rhythm of a regulated hypothermia in Japanese quail but never explored the effects of toxicants on the phenomenon. This study's use of radio telemetry implants to monitor the sublethal effects of OP exposure on thermoregulatory and cardiac function in birds therefore appears to be the first published application of telemetry for this purpose with the potential to both yield empirical data about the sublethal affects of OP pesticides on birds and demonstrate the feasibility and utility of using telemetry in avian studies of this nature.

This study uses radio telemetry implant techniques to test the hypothesis that sublethal oral exposure to OPs can cause effects in cardiac function and thermoregulation.

6.2 MATERIALS AND METHODS

6.2.1 Experimental Species

Coturnix quail (*Coturnix coturnix*) were purchased from Fircrest Farms Ltd. (Langley, BC, Canada). All quail used in the study were males between 6 and 26 weeks of age.

6.2.2 Animal Care and Housing

The study was conducted in accordance with Protocol #20070071 approved by the Research Ethics Board of the University Committee on Animal Care and Supply at the University of Saskatchewan. All quail were housed indoors at the Animal Care Unit in the Western College of Veterinary Medicine, University of Saskatchewan, from their date of arrival until surgical implantation of radio telemetry devices. After surgery, quail were transferred to the Toxicology Centre at the university during the post-surgical recovery period and held within well-ventilated individual containers in an anteroom to an environmental chamber. Quail were maintained under a 8 hours light:16 hours dark cycle and were provided a commercial, medicated (amprolium 125 mg/kg), crumbled turkey starter feed (Federated Co-operatives Limited, Saskatoon, SK, Canada) and water *ad libitum*.

6.2.3 Experimental Design

A completely randomized design was used to assess the effect of sublethal oral organophosphate pesticide exposures on heart rate and body temperature. Quail were assigned to three groups; sublethal oral exposure to two different OP pesticides and one sham. Heart rate and body temperature were measured in each quail repeatedly over a five day period both before and

after exposure and under both simulated day-time thermoneutral and night-time cold-stressed conditions. The sample size was five birds per trial.

6.2.4 Organophosphate Insecticide Exposures

Spray formulations of dicrotophos (DCP)(BIDRIN®, 82.0% DCP by weight, Amvac Chemical Corporation) and ethoprop (ETP)(MOCAP®, 69.6% ETP by weight, Bayer CropScience) were used in the experiment. These formulations were diluted in reverse osmosis water as needed and quail were dosed by oral gavage at approximately one-third the estimated LD_{50} for this species. Oral dose rates were 1.9 and 2.5 mg/kg for DCP and ETP, respectively. Additional trials were completed with the OPs methamidophos and naled but these efforts are not reported here (Supplemental Material).

6.2.5 Radio Telemetry Equipment and Surgeries

Five battery-free implantable transponders (G2-HR E-Mitter), a single energizer/receiver (ER-4000 Energizer/Receiver), and related software (VitalView Data Acquisition Software) were purchased from Mini Mitter Company Inc (Bend, OR, USA).

Quail were anaesthetized by isoflurane inhalation and prepared for sterile surgery. The temperature sensors were placed subcutaneously in the abdominal wall along the fold formed by the inner thigh and belly on the right side. The electrodes were located over the heart and attached to breast muscle with metal suture wire. Following recovery from anaesthesia, quail were housed in individual containers and placed under observation for a minimum of 1 week prior to data collection. All implanted quail recovered from surgery without complication.

The battery-free transponders had to be held immediately above the energizer/receiver to be energized and so collect and transmit data. The use of a single energizer/receiver for the five transponders required rotating implanted quail on and off of the energizer/receiver. This was accomplished without direct physical contact with the quail by moving entire individual housing containers.

It was necessary to reuse the implants for each trial. Implants were removed from the quail carcasses following euthanasia, cleaned and gas-sterilized using ethylene oxide. Data were collected in the various trials in sequence.

6.2.6 Body Temperature and Heart Rate Data Collection

Data were collected on 4 days over a 5 day protocol: Day -1 (one day pre-exposure); Day 0 (exposure day); Day 1 (one day post-exposure); and Day 3 (three days post-exposure). The sham and sublethal pesticide exposures were administered by oral gavage between 1000 and 1100 hours on Day 0. The gavages were given to the telemetred quail in sequence, one every twelve minutes, over the one hour exposure period. The sham consisted of vehicle alone at volumes similar to those used to deliver the pesticides. Discrete measures of body temperature and heart rate data were collected hourly under simulated day-time thermoneutral conditions (18-21°C) from 1300 to 1600 hours and under simulated night-time cold-stressed conditions (4-5°C) from 1700 to 2100 hours on each of the four observation days. Data were collected from the telemetred quail sequentially, one quail every twelve minutes, in a manner consistent with their dosing. Observations under thermoneutral conditions were recorded in the anteroom to the environmental chamber where quail were held for post-surgery recovery. Observations under cold-stressed conditions were made within the environmental chamber itself.

6.2.7 Statistics

All data used in statistical analyses were normalized to a zero-mean on the pre-exposure day (Day -1). Data were examined using repeated measures analysis of variance (ANOVA). *Post hoc* multiple comparisons were completed using paired t-tests with Bonferroni corrections. The relationship between effects on thermoregulation and effects on heart rate were investigated using ordinary least squares regression. Significance was associated with $p<0.05$. All statistical analyses were completed on PASW Statistics 18.0 software (SPSS Inc.).

6.3 RESULTS

6.3.1 Body Temperature and Heart Rate in Unstressed Quail

Body temperature and heart rate in quail under thermoneutral conditions on the pre-exposure day were assumed representative of an unstressed condition. Differences in body temperature and heart rate among trials existed but were not significant and observations were pooled. Mean (SE) body temperature and heart rate were 41.0°C (0.4°C)(n=14) and 242 beats per minute (bpm)(28 bpm)(n=14), respectively.

6.3.2 Sample Losses

One quail in the ETP-exposed group was removed from cold-stressed conditions on Day 0 when its body temperature dropped to 36.3°C. This bird was ataxic with shivering or tremors and drooped wings when removed from the environmental chamber and it died later that night.

6.3.3 Thermoregulation

The multiple hourly discrete measures of body temperature obtained during observations in each of the day-time thermoneutral and night-time cold-stressed conditions were used to generate single measures of body temperature for each quail in each temperature regime on each day. Body temperatures were generally consistent throughout the day-time thermoneutral observations and the average of all discrete measures collected during each daily thermoneutral period served as the daily single measure of body temperature under thermoneutral conditions (T_t). Body temperatures declined to a stable level during the first couple of hours of observations under night-time cold-stressed conditions and the average of the last two hourly measures of body temperature served as the daily single measure of body temperature under cold-stressed conditions (T_c).

The effect of day on T_t in the sham was not significant. Both OP trials showed a significant effect of day (DCP, F_3=15.15, $p<0.001$; ETP, F_3=9.45, $p=0.004$). *Post hoc* tests were performed on data in the OP trials to examine differences between effects among days (Figure 6.1). Hypothermia was evident in both the DCP and ETP quail on Days 0 and +1.

T_c data were analyzed in a manner consistent with that used for T_t. No significant effect of day was found in any trial.

6.3.4 Heart Rate

Some quail were clearly agitated by the environmental chamber as indicated by increases in heart rate upon entry into the chamber. Noise from the compressor in the environmental chamber was suspected to cause stress in quail within the chamber. This stress is also clearly not

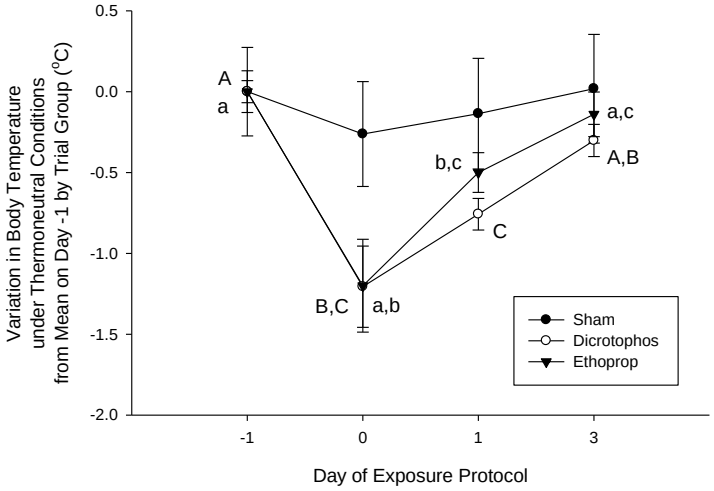


Figure 6.1. Variation in body temperature under thermoneutral conditions (T_t) in sham-exposed and organophosphate-exposed quail among days where days are: -1) one day pre-exposure; 0) the day of exposure; 1) one day post-exposure; and 3) three days post-exposure. Error bars denote (+/-) standard error. Upper and lower case letters denote homogenous subsets within the dicrotophos and ethoprop trials, respectively ($p<0.05$).

related to OP-exposure as it was observed in some sham-exposed quail and in some pesticide-exposed quail in the pre-exposure days. Heart rate was also elevated in response to handling of the housing containers but rapidly returned to normal.

The effect of day in HR_t data of the sham trial was not significant. The DCP and ETP trials both showed highly significant effects of day (DCP, $F_3=22.53$, $p<0.001$; ETP, $F_3=26.52$, $p<0.001$). *Post hoc* tests revealed differences between effects among days (Figure 6.2). In both cases that response was an ephemeral tachycardia apparent on Day 0.

Analysis of HR_c data in the ETP trial revealed an effect of day ($p=0.035$). However, as *post hoc* tests failed to find any differences between HR_c on Day 0 and any other day in the ETP trial, the results were inconclusive.

6.3.5 Linear Regressions

Regression using measures of the change in HR_t and change in T_t (both Day 0 minus Day -1) in quail from all treatments (n=14) revealed a significant negative correlation between the parameters (F_1=9.264, p=0.010)(Figure 6.3). Variation in the change in HR_t explained approximately 44% of the variation in change in T_t. The intercept was -0.401°C but not significant. A similar regression using change in both HR_c and T_c failed to find a relationship between these variables.

6.3.6 Observed Thermoregulatory Failure and Mortality

Observations in three quail excluded from statistical analyses are exceptional in that the hypothermia observed in these quail was extreme and suggests an almost complete failure in thermoregulatory ability in response to cold stress. T_t in the ETP-exposed quail that died following data collection on the day of exposure was 37.0°C. Hourly body temperature data indicate that this quail was actually recovering from an extreme hypothermia in the first few hours immediately following dosing. At 7 hours post-dose when the quail was introduced into the environmental chamber its body temperature was 38.3C°. One hour later it was removed from the environmental chamber with a body temperature of 36.3°C. The maximum heart rate observed in this bird was 533 bpm, a tachycardia of 291 bpm relative to the mean unstressed heart rate. Two quail from a preliminary trial with naled had obviously compromised thermoregulatory abilities that persisted into Day +3. Both of these quail had to be rescued from the cold chamber in Days 0, +1 and +3. On Day 0, the lowest observed body temperatures in these quail were 37.0 and 36.9°C. The extreme in heart rate on the day of exposure was 563 bpm

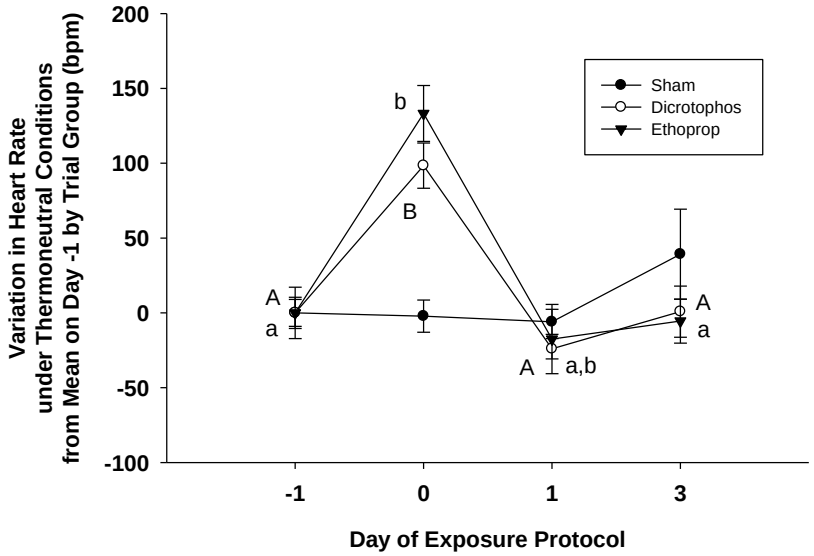


Fig. 6.2. Variation in heart rate under thermoneutral conditions (HR_t) in sham-exposed and organophosphate-exposed quail within days where days are: -1) one day pre-exposure; 0) the day of exposure; 1) one day post-exposure; and 3) three days post-exposure. Error bars denote (+/-) standard error. Upper and lower case letters denote homogenous subsets within the dicrotophos and ethoprop trials, respectively ($p<0.05$).

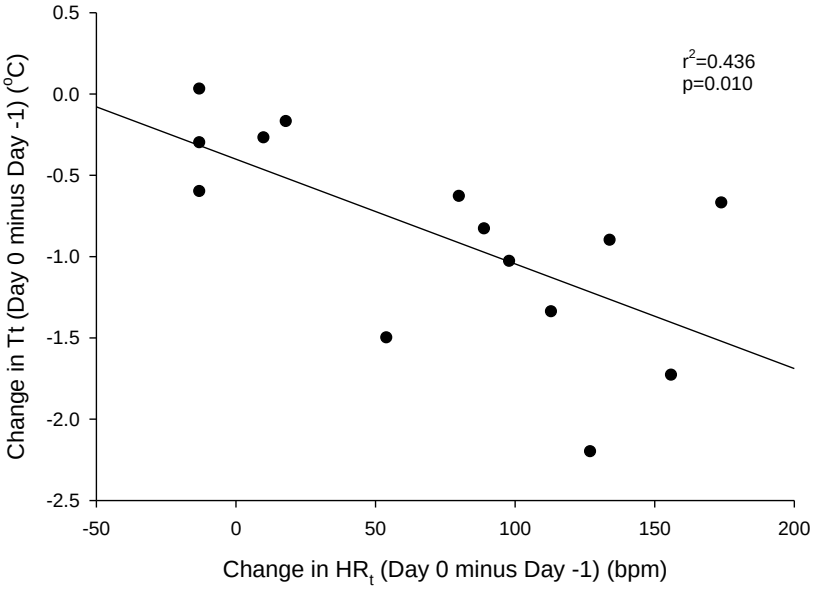


Fig. 6.3. Linear regression between effects in heart rate and thermoregulation in quail exposed to dicrotophos using change in heart rate under thermoneutral conditions (HR_t) and change in body temperature (T_t) from the day of exposure (Day 0) to the pre-exposure day (Day -1)(n=14).

in one bird. The thermoregulatory abilities of these two quail in response to cold-stress were compromised for 80 hours post-dose when the experiment was terminated.

6.4 DISCUSSION

Quail exposed to DCP and ETP presented with significant hypothermia of 1.21 and 1.20°C and significant tachycardia of 98.4 and 133 bpm, respectively, under thermoneutral conditions in a period 3 to 6 hours post-dose on the day of exposure relative to their condition during the same time period on the pre-exposure day. The observed hypothermia in the DCP and ETP quail persisted into the first post-exposure day although the effects, 0.76 and 0.50°C, respectively, were reduced. Effects on heart rate in these birds had dissipated by 1 day post-exposure. Effects on thermoregulation persisted for longer than effects on heart rate.

Although significant hypothermia was evident in both the DCP and ETP quail under thermoneutral conditions on Days 0 and +1, no significant hypothermia was observed in the intermediate period (i.e. under cold-stressed conditions on Day 0) for either compound. A probable confounding factor is the presence of a diurnal regulated hypothermia. Underwood (1994) examined the circadian rhythm of thermoregulation in telemetred Japanese quail, a species closely related to the Coturnix quail utilized in this work, and found them to possess a circadian rhythm in regulation of body temperature that was strongly entrained to light-dark cycles. We postulate that a similar diurnal regulated hypothermia exists in our species and that it and any regulated hypothermia in response to the OP insult and any unregulated hypothermia in response to cold-stressed conditions were not additive but coincident. This suggests that hypothermia may have limited application as a measure of OP effects on thermoregulation under cold-stressed conditions.

Quail were obviously stressed by handling and many were sensitive to the environmental chamber in which measurements of HR_c were made. Heart rate in these quail was elevated in response to these stimuli. Quail in the DCP and ETP trial did display tachycardia under cold-stressed conditions on the day of exposure, 64.8 and 102 bpm relative to the pre-exposure condition, respectively, but this response was highly variable and statistical analyses were inconclusive.

The coincidence of effects in heart rate with effects on thermoregulation is of interest as the cardiovascular and thermoregulatory systems are coupled. There is the possibility that OP effects on the cardiovascular system may interfere with thermoregulatory ability (i.e. prevent thermoeffector control over vasoconstriction/vasodilation).Whether or not the observed tachycardia is indicative of such effects is unknown. Nonetheless, the adverse effects on thermoregulation may have been exacerbated by dysfunction in the cardiovascular system.

These findings are important for several reasons. Firstly, the observations with DCP and ETP appear to offer only the second record of significant OP-induced effects on heart rate in birds and the first record using unrestrained telemetered birds. Secondly, the observations with these OPs describe a progression of effects on body temperature and thermoregulation with increasing OP toxicity ranging from a mild and potentially beneficial regulated hypothermia in response to OP-exposure under thermoneutral conditions to a severe dysfunction in thermoregulatory ability that is tolerable under thermoneutral conditions but potentially lethal under cold-stressed conditions. Lastly, this work demonstrates the utility of telemetry in observing sublethal effects on the physiology of birds in response to OP-exposure.

It may be that the use of telemetred birds in pen studies could finally provide the answers to the questions posed by Heinz (1989). However, it is also apparent that further laboratory work

is necessary to better describe how sublethal effects progress and potentially interact in causing mortality. Although interactions between anticholinesterase toxicity and thermal stresses have been observed (Rattner et al. 1987, Martin and Solomon 1991), the mechanisms involved in these interactions are at best poorly understood.

Chapter 7

7.0 SUMMARY AND CONCLUSIONS

7.1 CONCLUSIONS

The research objectives and related conclusions are:

1. To determine the contributions of various routes of exposure to total toxicity in birds from spray applications of a set of OP pesticides of varying physicochemical properties under simulated field conditions.

The dermal route of exposure was the primary contributor to total toxicity in quail under simulated field conditions regardless of the exposure scenario tested, the OP pesticide used, or the post-exposure period at which toxicity was determined. The oral route of exposure contributed little to toxicity either as quail were deterred from feeding, as was the case with MMP and NLD (thought due to their irritant properties), or as the feed consumed produced no significant effect in brain, as was the case with DCP and ETP (thought due to the sheltering effect of the vegetative canopy limiting contamination of the feed). Consumption of feed on the simulated field failed to explain the toxicity observed in brain. Exposure to the pesticide spray itself produced significant increases in toxicity as measured in plasma (DCP, ETP, and MMP) and brain (NLD). Differences in inhibition and recovery of ChE activity in quail between control oral gavage exposures and simulated field exposures suggest the presence of a skin reservoir for storage and delayed release of dermally absorbed OPs. The effect of this reservoir increased with increasing lipophilicity of the OP, consistent with a phenomenon previously described in studies with mammals.

2. To develop an exposure model for predicting the contributions of the oral, dermal, and potentially other routes of exposure to total toxicity of OP pesticide sprays in birds based upon the physicochemical properties of the OP itself and its application rate.

Log K_{ow} of the OPs significantly predicted inhibition of brain ChE activity in simulated field exposures at both 24 and 72 hours post-exposure and also significantly predicted further inhibition of brain ChE activity associated with additional direct exposure to the pesticide spray itself, although this relationship was relatively weak. Quadratic polynomial curves were fit to the data in each case. The findings predict that directly acting OPs of log K_{ow} in the range of 0 to 1.5 are those most readily absorbed and distributed via the dermal route under field conditions. These findings are generally consistent with observations in mammals.

3. To determine if short term dehydration of birds confounds the diagnosis of OP-exposure using measures of ChE activity in brain or plasma.

Short term dehydration resulting from water deprivation over a period of 5 days caused an apparent increase in plasma ChE activity in quail. This effect in quail was antagonistic to that which resulted from a sublethal oral dose of dicrotophos (one-third the LD_{50}). The two stressors in combination produced plasma ChE activity that appeared slightly induced, although it was not significantly different from that of control quail. Under these circumstances, dehydration confounded the diagnosis of dicrotophos exposure using plasma ChE activity. A method for correcting the observed plasma ChE activity for the effects of dehydration using measures of P_{osm} and the observed relationship between P_{osm} and plasma ChE activity was developed.

Application of this method to the data provided a more robust portrayal of dicrotophos exposure and eliminated the confounding affect of dehydration.

4. To determine if sublethal oral exposure to OPs adversely affects cardiac and thermoregulatory function in birds under different temperature regimes (18°C and 4°C) using radio telemetry implants.

Sublethal oral doses of DCP and ETP at one-third the estimated LD_{50} caused significant hypothermia and tachycardia in quail as measured using telemetry implants. These effects were ephemeral and evident only in the day immediately following the exposure under thermoneutral conditions. Analyses of brain ChE activity of other similarly exposed quail suggest that telemetred quail exposed to DCP and ETP had greater than 50% inhibition of brain ChE activity in the day following exposure. The effects on body temperature and heart rate in quail using doses of MMP and NLD were not significant and this was attributed to a much lower inhibition of brain ChE activity in these birds. Observations in three quail excluded from statistical analyses suggest that effects on thermoregulation which are sublethal under thermoneutral conditions can become lethal under cold-stressed conditions. The effects observed in these three quail included an extreme ephemeral tachycardia and a failure of thermoregulatory ability under cold-stressed conditions that persisted to the end of the protocol at three days post-exposure.

7.2 CONTRIBUTIONS

1. This appears to be one of few published uses of measures of effect size in ANOVA with avian cholinesterase data. A literature search using the Google Scholar search engine using

the key words ‘birds cholinesterase’ first, ‘birds cholinesterase ANOVA’ second, and ‘birds cholinesterase ANOVA eta-squared’ lastly produced ~10,300 results, ~1,170 results, and 3 results, respectively. None of the 3 results found in the last search were actually related to avian cholinesterase research. Similar searches with academic search engines (TOXLINE, PubMed, CABI, Scopus) were even less fruitful. Although eta-squared first appeared in the literature in 1911 (Pearson 1911) and although practitioners in other fields in past decades have both espoused the utility of eta-squared and partial eta-squared and offered direction on how to conduct such analyses (Cohen 1973, Cohen 1988, Levine and Hullett 2002), researchers studying the effects of anticholinesterase pesticides on birds appear ignorant of the utility of measures of effect size. This may simply reflect a lack of complex experimental designs in this field. Regardless, the author offers his own results in dealing with significant effects in pre-exposure plasma ChE activity as evidence in seconding the opinion of Levine and Hullett (2002) that “there are good reasons to report estimates of effect size in addition to p-values”.

2. This appears to be the first instance in which cholinesterase activity as measured in unknowns, here the brains of Coturnix quail, has been normalized to the median activity in a highly variable in-house cholinesterase assay analytical standard. Rather it is common practice to reject and repeat cholinesterase assays when activity in the standard falls outside a narrow range about the mean. Indeed the author would have preferred such circumstances in this work. Nonetheless, the method developed for normalizing activity in unknowns to a measure of central tendency in the in-house standard may prove useful to others as the evidence presented here suggests the method has practical benefits. Firstly, variability in the activity of an in-house analytical standard may be more tolerable than is generally believed

and this ‘noise’ may be better interpreted as a ‘signal’ upon which one can base corrections to observations in unknowns. The result is that time and materials need not be wasted on the unnecessary repetition of cholinesterase assays and this has obvious benefits in the saving of money, sample, and frustration. Secondly, it appears that this method resulted in an estimate of reference brain cholinesterase activity among the most precise published in the primary literature with a comparable sample size. This new use of the in-house standard appears to be an improvement to current practice in estimating brain cholinesterase activity in birds.

3. An influence of the dermal route of exposure on the toxicodynamics (inhibition and recovery of ChE activity in plasma and brain) to spray applications of DCP, NLD, and ETP under simulated field conditions was demonstrated. Dermal absorption appeared to be the main contributor to the toxicity observed under simulated field conditions. Although differences in the toxicodynamics of the oral and dermal routes of exposure routes have been demonstrated under laboratory conditions with controlled exposures (i.e. oral gavage and solvent-deposited dermal doses) with both mammals (Kramer et al. 2002b) and birds (Henderson et al. 1994), this is the first instance in which the influence of route of exposure on toxicodynamics has been observed with exposures approximating those expected under field conditions (i.e. with DCP and ETP). Further, the inferences made herein differ from those of Driver et al. (1991), who also inferred dermal exposure as being the primary contributor to the toxicity of an OP under simulated field conditions, in that these inferences were made based upon observations resulting from experiments with a set of OPs of varying solubility properties and in that a single mechanism was proposed to explain both the common influence of the dermal route of exposure on simulated field exposures among DCP, NLD, and ETP collectively and the differences in the toxicodynamics of the simulated field exposures between them. This

mechanism, the reservoir effect, is well-documented in research on transdermal drug delivery in humans (Roberts et al. 2004) but its relevance in avian ecotoxicology is uncertain. The evidence here indicates that the formation of a pesticide reservoir in avian skin is of relevance to avian ecotoxicology.

4. This is the first demonstration that the solubility properties of OPs influence the absorption, distribution, and toxicity (i.e. inhibition of ChE activity) of an OP following pesticide spray application under conditions approximately those in the field. Herein, all else being generally equal, OPs with balanced lipid:water solubility appeared to be more hazardous to quail than OPs more extreme in their solubility properties. Further, this finding was generally consistent with prior work (Mineau 2002) that found adding a term integrating several physicochemical properties of anticholinesterase pesticides into logistic regression models of mortality risk based upon a field study data set significantly improved model performance. The term added to the models was negatively correlated with log K_{ow} of the active ingredient, implying anticholinesterases of lower log Kow (i.e. balanced lipid:water solubility) were more hazardous in the field than more lipophilic anticholinesterases. Collectively, the work here and that of Mineau (2002) indicate the current screening level pesticide risk assessment model (i.e. the quotient method) could be improved by giving consideration to the physicochemical properties of the active ingredient in pesticide sprays using OPs specifically and anticholinesterases generally.
5. The effect of dehydration on quail as observed in measures of P_{osm} and Hct and the consequent significantly related variation in plasma ChE activity suggest that variation in the water balance within birds explains some of the known variation in plasma ChE activity. This is the first physiological mechanism proposed to explain variation in plasma ChE

activity and, as such, is important given the high degree of variability known in this parameter. Indeed the utilization of plasma ChE activity in the investigation of anticholinesterase exposure and effects is diminished by its highly variable nature. The advice to clinicians and researchers taking measures of plasma ChE activity is therefore simply this: You have covariates in P_{osm} and Hct and you should measure them! Admittedly, the degree to which measures of P_{osm} and Hct prove useful in observational studies involving plasma ChE activity in wild birds is unknown. If the bird population under study has easy access to water, then measures of P_{osm} and Hct may be of little benefit. However, the potential for variation in the water balance as measured with P_{osm} and Hct, surrogates for the ECV and PV, respectively, to explain the presence of outlier measures of plasma ChE activity or relate autocorrelated variation (i.e. diurnal, seasonal) in plasma ChE activity to behaviour, feeding strategies, and potentially life history traits is undeniable. Indeed, as there is evidence that suggests variation in the water balance in birds may be part of a generalized short term stress response (Olanrewaju et al. 2007), then monitoring the water balance in birds may reveal relations between plasma ChE activity and stressors other than water deprivation, including toxicity from xenobiotics other than anticholinesterases, infection and disease, and injury.

6. The utility of telemetry techniques in the investigation of anticholinesterase exposure-induced effects on the cardiovascular and thermoregulatory systems of birds was demonstrated. Telemetrey implants designed for use with laboratory rodents were adapted for use in quail. The observations of concurrent significant tachycardia and hypothermia in quail resulting from anticholinesterase exposure are the first such records using telemetred birds. These records also describe a progression of effects from mild and not significant to strong

and significant dysfunction in the cardiovascular and thermoregulatory systems which in three quail were at once tolerable under thermoneutral conditions but potentially lethal under cold-stressed conditions.

7.3 FUTURE RESEARCH

7.3.1 Use of In-House Cholinesterase Assay Analytical Standards

The in-house analytical brain cholinesterase assay standard used in this work provided a means to correct observations in brain unknowns for unwanted effects thought to result primarily from between-batch variation in the assay temperature. The end result was a measure of control quail brain ChE activity that was remarkable in its precision given the variability in the standard. Although the utility of this method is evident in these results, the method has not been subject to a rigorous validation study. Therefore, the degree to which this method will prove useful where the source of error is between-batch variability in assay temperature or some other factor (i.e. between-batch variability in reagent solutions) or prove useful with other tissues (i.e. plasma ChE) is deserving of further investigation.

Equation 5.1 may be rewritten as:

$$ {}^{std}A_{Ti}/{}^{std}A_{Tj} = {}^{unk}A_{Ti}/{}^{unk}A_{Tj} \qquad \text{(Eq. 7.1)} $$

where ${}^{std}A_{Ti}$ = ChE activity in the standard at a given temperature, Ti

${}^{std}A_{Tj}$ = ChE activity in the standard at some other temperature, Tj

${}^{unk}A_{Ti}$ = ChE activity in an unknown at a temperature, Ti

${}^{unk}A_{Tj}$ = ChE activity in the same unknown at temperature, Tj.

Hypothetically, this equation may be extended to include fixed concentrations of the reagent**,** Ri, and of the substrate, Si, of the Ellman ChE assay as follows:

$$^{std}A_{TiRiSi}/^{std}A_{TjRiSi} = {}^{unk}A_{TiRiSi}/^{unk}A_{TjRiSi.} \quad \text{(Eq. 7.2)}$$

One can then envision an investigation of how variation in assay temperature and concentrations of reagent and substrate solutions, each alone and in all possible combinations, influence measures of ChE activity in a single set of unknowns (including one unknown as a standard) using a factorial experimental design in which there are three factors, assay temperature and concentrations of the reagent and the substrate. Analyses would proceed using both RTO and ANOVA. RTO would be used to test the null hypothesis that there is no relationship between the different measures of activity in the unknowns and the standard resulting from variation in one or more of the factors, alone or in combination, here for example assay temperature and concentration of the reagent:

$$H_o: \quad \text{slope} = {}^{std}A_{TiRi}/^{std}A_{TjRj} = {}^{unk1}A_{TiRi}/^{unk1}A_{TjRj} = {}^{unk2}A_{TiRi}/^{unk2}A_{TjRj} = \ldots \ldots = 0 \quad \text{(Eq. 7.3).}$$

Further, ANOVA would be used to compare the mean activity in the unknowns, $\bar{A}$, in each treatment (different levels of the factors in all combinations) once normalized to the activity of the standard, $^{std}A_{TiRiSi}$, and so test the null hypothesis:

$$H_o: \quad \bar{A}_{TiRiSi} = {}^{norm}\bar{A}_{TjRiSi} = {}^{norm}\bar{A}_{TiRjSi} = \ldots \ldots = {}^{norm}\bar{A}_{TjRjSj} \quad \text{(Eq. 7.4).}$$

This effort would provide a statistically rigorous validation of the method developed for normalizing the activity in unknowns to a measure of central tendency in the standard.

The use of this method in the measurement of ChE activity in avian plasma is a more complicated matter. Avian plasma typically includes both AChE and BChE whereas the method developed in this thesis relies on the in-house standard being the same single enzyme as is being measured in the unknowns and so being similarly sensitive to variation in assay temperature and other factors contributing to variability in the assay. An extension of this method to plasma, if possible, will rely on the use of a selective inhibitor to discriminate between the activity

associated with each of the two enzymes, AChE and BChE. Thereafter, the experimental design described above may provide some insight. Such an investigation would also obviously require the use of two standards.

7.3.2 Dermal Exposure of Birds to Pesticides

This work indicates that the dermal route of exposure contributes to the toxicity of OP insecticidal sprays in birds in the field. However, the evidence presented here is indirect in that it relies on inhibition and recovery of ChE activity in tissues, biomarkers of exposure and effect, and not on direct measures of the amount of the compounds being absorbed through skin. Given the lack of direct evidence that the observed effects are the result of doses absorbed through skin, the inferences made may be doubted. It is therefore the author's opinion that a necessary next step is to better characterize dermal absorption in birds and how it varies with the physicochemical properties of the compound of interest.

Numerous methods have been developed to investigate dermal absorption in mammals due to interest in occupational exposure to pesticides, cosmetics, and transdermal drug delivery. Both the Organisation for Economic Co-operation and Development (OECD) and the EPA have developed guidelines for the *in vitro* and *in vivo* assessment of dermal absorption (van de Sandt et al. 2004). An investigation into how intra- and inter-laboratory variation affected predictions of skin absorption in compounds of varying physicochemical properties found that the standardized methods employed by the OECD yielded robust predictions of dermal absorption (van de Sandt et al. 2004). These established, standardized, and validated methods for dermal absorption studies in mammals can likely be adapted for use with birds. It would then be possible to confirm experimentally how dermal absorption in birds is similar to and different from dermal

absorption in mammals. For example, the observations herein indicate that compounds of balanced lipid:water solubility are more readily absorbed through bird skin than compounds that are either more hydrophilic or more lipophilic but no inference can be made as to whether the absorption rates were slower or faster than rates observed in mammals. Initial efforts could be focused on *in vitro* methods (i.e. diffusion cells) and non-toxic reference compounds of varying properties, like those recommended by the OECD (i.e. benzoic acid, caffeine, testosterone) (van de Sandt et al. 2004), thereby avoiding at least some of the issues related to animal welfare and hazardous materials handling that complicated this work. As with investigations using mammals, efforts could be focused on obtaining measures of maximum rate of absorption, time to maximum rate of absorption, and the mass balance (i.e. recoveries in donor and receptor solutions and in isolated skin samples) (van de Sandt et al. 2004) for comparisons among compounds and between species.

7.3.3 Modeling of Exposure and Effects

The intent of this work was to provide insight into what routes of exposure contribute to the toxicity of OP insecticidal sprays in birds in the field and subsequently develop methods for improving the accuracy in predictions of effects. This work therefore indirectly tested one of the central assumptions inherent in the QM of screening level avian ERA for pesticide registration: that alimentary exposure is the only route of exposure of relevance in assessing the risk to birds from pesticide use. This study indicates otherwise. The author's own opinions on the matter are that exposures in the field are extremely complex and involve multiple routes of exposure concurrently. Assuming complex, multiroute exposures, then a logical next step is to question another central assumption in avian pesticide effects studies: that effects among exposure routes

are additive. This assumption is well-enough embraced to have been adopted in experimental methods, those used for estimating toxicity resulting from dermal exposure in the work of Driver et al. (1991), for example, yet to the author's knowledge it has never been validated. The author's interest is therefore in a tool for modeling exposure and effects that is capable of handling the complexity of multiroute exposures and that does not rely on the assumption of additivity in effects among exposure routes.

Physiologically-based, pharmacokinetic, pharmacodynamic (PBTKTD) modeling offers exactly such a tool. There appear to be few avian PBTKTD models published in the primary literature. A literature search using the Google Scholar search engine and the key words 'birds physiologically based pharmacokinetic pharmacodynamic model' produced approximately 2,790 results. A review of the first 100 results found only 5 publications actually involving avian physiologically-based pharmacokinetic models, none of which included pharmacodynamic parameters. Four of the 5 were studies on drug residues in poultry intended for human consumption and the fifth was on the accumulation of persistent organic pollutants in avian wildlife. The author knows of no published avian PBPKPD models for OP exposure although such models have been developed for mammals (Timchalk et al. 2002). The interest in PBPKPD models lies in the ability of such models to combine doses from different routes in estimating the effects of complex multiroute exposures (Tan et al. 2006). Further, these models provide for the reconstruction of exposures from biomarker data (Mosquin et al. 2009), an modeling exercise analogous to the reasoning applied in Chapter 3 where patterns of recovery of ChE activity in tissues were used to make inferences about the routes of exposure contributing toxicity.

7.3.4 Stressors and Variability in Plasma Cholinesterase Activity in Birds

The observation that dehydration induced by deprivation of water caused an apparent increase in plasma ChE activity implies that changes in the body water balance of birds generally, regardless of cause, might contribute to substantially to the known high degree of variability in this parameter. As the high degree of variability in plasma ChE activity reduces its utility as a measure of anticholinesterase exposure, any effort that characterizes or explains this variability is bound to be of utility.

It will be prudent to collect measures of P_{osm} and Hct concurrently with measures of plasma ChE activity and treat them as covariates in statistical analyses in observational studies conducted in the field and so attempt to differentiate between affects related to variation related to maintenance of water balance from affects related to anticholinesterase exposure. Both analysis of covariance and multivariate ANOVA are statistical tools that could be applied in field studies comparing plasma ChE activity in birds on reference and exposure sites.

Of greater interest to the author, however, is the potential for stress generally, or at least some other stressors in addition to water deprivation, or behaviour, or feeding strategies, etc. to explain variation in avian plasma ChE activity. Fundamentally, the author questions whether or not the high degree of variability inherent in plasma ChE activity can be placed in a physiological and ecological context and not simply characterized with respect to species, date, time, and place. Hence efforts to characterize the variability of plasma ChE activity in some species in some location based upon season and time, as has been and remains practice, although undeniably informative, is necessarily delimited by the species, place, and time of the investigation and therefore of limited utility in making predictions in other species, places, and times.

Among the questions of interest are: Does stress in birds generally result in changes in the avian water balance that indirectly also cause changes in apparent plasma ChE activity? Are there multiple stressors that can confound the diagnosis anticholinesterase exposure using plasma ChE activity via their effect on water balance? These questions are prompted in part by the observations of Olanrewaju et al. (2007). They induced physiological stress in broiler chickens with implanted osmotic pumps continuously delivering adrenocorticotropic hormone into circulation, observed both significant losses of body weight and significant increases in P_{osm} following seven days of treatment, and then inferred loss of body water and difficulty in maintenance of the water balance as resulting from the induced stress (Olanrewaju et al. 2007). Indeed the author is interested in expanding upon the work of Olanrewaju et al. (2007) but with an additional focus on the effects on plasma ChE activity.

7.3.5 Telemetry and Observations of Sublethal Effects

Constraints on budget and the small size of the quail imposed limitations on the type and quantity of experimental work that could be performed using telemetry equipment. Quail were too small to receive battery-powered implants and instead received implants containing copper coils which when implanted quail were placed within an electrical field produced by the receiver were then energized and capable of collecting and transmitting data. Only five implants and a single energizer/receiver were purchased as these materials were more expensive than anticipated. The result was that collection of data from implanted quail was labour intensive as quail had to be rotated on and off the energizer/receiver in sequence. The full potential of telemetry equipment was not realized in this work. Nonetheless, the data obtained and the experience gained did give the author sufficient information to postulate a mechanism that might

explain how effects in the cardiovascular and thermoregulatory systems that are tolerable under thermoneutral conditions can become lethal under cold-stressed conditions.

The cardiovascular and thermoregulatory systems of birds and mammals are coupled. Thermosensors perceive cold-stress and then cue thermoeffectors to respond. Among the responses produced is vasoconstriction within the cardiovascular system. Vascoconstriction reduces blood flow to peripheral tissues and thereby reduces heat loss under cold-stress. The three quail which exhibited failure in thermoregulatory ability under cold-stressed conditions were all identified by a continuous drop in body temperature as time passed under cold-stressed conditions. In one quail this was lethal and in the other two it prompted their rescue from cold-stress conditions in all three post-exposure days of the study protocol. These quail evidently could not prevent heat loss from their bodies. Is this simply a matter of these quail, at least to some degree, being unable to respond to cold-stress with vasoconstriction? Does anticholinesterase exposure at sufficient dose eventually cause the uncoupling of the thermoregulatory and cardiovascular systems that leads to thermoregulatory failure under cold-stressed conditions? Such questions are relevant if anticholinesterase pesticides are used on crops in temperate climates where night time temperatures impose cold-stress on wildlife.

There exist radio telemetry implants capable of simultaneous measurements of blood pressure, body temperature, and electrical activity in the heart and brain (i.e. those of TSE Systems, Inc., Chesterfield, MO, USA). These implants are intended for use with larger laboratory mammals (i.e. body mass of 170 g or more) in group housing and could therefore likely be adapted for use with waterfowl or game birds in pen studies. The use of such implants with birds in pen studies would allow the sublethal effects of anticholinesterase pesticides on

aspects of avian physiology to be investigated and potentially provide insight into the conditions under which sublethal effects can become lethal and the underlying mechanisms involved.

REFERENCES

Akamatsu M. 2011. Importance of physicochemical properties for the design of new pesticides. *Journal of Agricultural and Food Chemistry* 59: 2909-2917.

Aldridge WN and Davison AN. 1953. The mechanism of inhibition of cholinesterases by organophosphorous compounds. *Biochemical Journal* 55: 763-766.

Arad Z, Arnason SS, Chadwick A, and Skadhauge E. 1985. Osmotic and hormonal responses to heat and dehydration in the fowl. *Journal of Comparative Physiology B* 155:227-234.

Arad Z, Horowitz M, Eylath U, and Marder J. 1989. Osmoregulation and body fluid compartmentalization in dehydrated heat-exposed pigeons. *American Journal of Physiology* 257: R377-R382.

Barnthouse L. 2008. The strengths of the ecological risk assessment process: linking science to decision making. *Integrated Environmental Assessment and Management* 4: 299-305.

Bascietto J, Hinckley D, Plafkin J, and Slimak M. 1990. Ecotoxicity and ecological risk assessment: regulatory applications at EPA. *Environmental Science and Technology* 24: 10-15.

Benson HAE. 2012. Skin structure, function, and permeation. In Benson HAE, Watkinson AC, eds, *Topical and transdermal drug delivery: principles and practice*, John Wiley & Sons, Inc., Hoboken, New Jersey, USA, pp 3-22.

Berteau PE and Deen WA. 1978. A comparison of oral and inhalation toxicities of four insecticides to mice and rats. *Bulletin of Environmental Contamination and Toxicology* 19: 113-120.

Blus LJ, Staley CS, Henny CJ, Pendleton GW, Craig TH, Craig EH, and Halford DK. 1989. Effects of organophosphorus insecticides on sage grouse in southeastern Idaho. *Journal of Wildlife Management* 53: 1139-1146.

Bronaugh RL, Stewart RF, and Congdon ER. 1982. Methods for in vitro percutaneous absorption studies II. Animal models for human skin. *Toxicology and Applied Pharmacology* 62: 481-488.

Brown MB, Turner R, and Lim ST. 2012. Topical product formulation development. In Benson HAE, Watkinson AC, eds, *Topical and transdermal drug delivery: principles and practice, 1st ed.*, John Wiley & Sons, Inc., Hoboken, New Jersey, USA, pp 255-286.

Burn JD and Leighton FA. 1996. Further studies of brain cholinesterase: cholinergic receptor ratios in the diagnosis of acute lethal poisoning of birds by anticholinesterase pesticides. *Journal of Wildlife Diseases* 32: 216-224.

Carmi N, Pinshow B, and Horowitz M. 1994. Plasma volume conservation in pigeons: effects of air temperature during dehydration. *American Journal of Physiology* 267: R1449-R14543.

Çetin N, Çetin E, Eraslan G, and Bilgili A. 2007. Chlorpyrifos induces cardiac dysfunction in rabbits. *Research in Veterinary Medicine* 82: 405-408.

Chapman PM, Fairbrother A, and Brown D. 1998. A critical evaluation of safety (uncertainty) factors for ecological risk assessment. *Environmental Toxicology and Chemistry* 17: 99-108.

Cherrie JW and Robertson A. 1995. Biologically relevant assessment of dermal exposure. *Annals of Occupational Hygiene* 39: 387-392.

Clark TP, Norstrom RJ, Fox GA, and Won HT. 1987. Dynamics of organochlorine compounds in herring gulls (*Larus argentatus*): II. A two-compartment model and data for ten compounds. *Environmental Toxicology and Chemistry* 6: 547-559.

Cleek RL and Bunge AL. 1993. A new method for estimating dermal absorption from chemical exposure. 1. General approach. *Pharmaceutical Research* 10: 497-506.

Cobos, V, Mora M, Escalona G, Calme S and Jiménez J. 2010. Variation in plasma cholinesterase activity in the clay-colored robin (*Turdus grayi*) in relation to time of day, season, and diazinon exposure. *Ecotoxicology* 19: 267-272.

Cohen J. 1973. Eta-squared and partial eta-squared in fixed factor ANOVA designs. *Educational and Psychological Measurement* 33: 107-112.

Cohen J. 1988. *Statistical Power Analysis for the Behavioral Sciences*. Lawrence Erlbaum Associates, Inc., Hillsdale, New Jersey, USA.

Cortright KA, Wetzlich SE, and Craigmill AL. 2009. A PBPK model for midazolam in four avian species. *Journal of Veterinary Pharmacology and Therapeutics* 32: 552-565.

Custer TW and Ohlendorf HM. 1989. Brain cholinesterase activity of nestling great egrets, snowy egrets and black-crowned night-herons. *Journal of Wildlife Diseases* 25: 359-363.

DeWeese LR, McEwen LC, Settimi LA, and Deblinger RD. 1983. Effects on birds of fenthion aerial application for mosquito control. *Journal of Economic Entomology* 76: 906-911.

Dodge JT, Mitchell C, and Hanahan DJ. 1963. The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. *Archives of Biochemistry and Biophysiology* 100: 119-130.

Dowling KC and Seiber JN. 2002. Importance of respiratory exposure to pesticides among agricultural populations. *International Journal of Toxicology* 21: 371-381.

Driver CJ, Ligotke MW, van Voris P, McVeety DB, Greenspan BJ, and Drown DB. 1991. Routes of uptake and their relative contributions to the toxicologic response of northern bobwhite (*Colinus virginianus*) to an organophosphate pesticide. *Environmental Toxicology and Chemistry* 10: 21-33.

Duke LD and Taggart M. 2000. Uncertainty factors in screening ecological risk assessments. *Environmental Toxicology and Chemistry* 19: 1668-1680.

Edge WD, Carey RL, Wolff JO, Ganio LM, and Manning T. 1996. Effects of Guthion 2S® on *Microtus canicaudus*: a risk assessment validation. *Journal of Applied Ecology* 33: 269-278.

Edge WD and Schauber EM. 2000. Factors affecting risk assessment of small mammals to pesticides. *Environmental Toxicology and Chemistry* 19: 2735-2741.

Eisenhauer J. 2003. Regression through the origin. *Teaching Statistics* 25: 76-80.

Elliot JE, Birmingham AL, Wilson LK, McAdie m, Trudeau S, and Mineau P. 2008. Fonofos poisons raptors and waterfowl several months after granular application. *Environmental Toxicology and Chemistry* 27: 452-460.

Ellman GL, Courtney KD, Andres J, and Featherstone RM. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochemical Pharmacology* 7: 88-95.

Fairbrother A, Bennett RS, and Bennett JK. 1989. Sequential sampling of plasma cholinesterase in mallards (*Anas platyrhynchos*) as an indicator of exposure to cholinesterase inhibitors. *Environmental Toxicology and Chemistry* 8: 117-122.

Fairbrother A, Craig MA, Walker K, and O'Loughlin D. 1990. Changes in mallard (*Anas platyrhynchos*) serum chemistry due to age, sex, and reproductive condition. *Journal of Wildlife Diseases* 26: 67-77.

Fildes K, Astheimer LB, Story P, Buttemer WA, and Hooper MJ. 2006. Cholinesterase response in native birds exposed to fenitrothion during locust control operations in eastern Australia. *Environmental Toxicology and Chemistry* 25: 2964-2970.

Fite E. 1994.The Environmental Protection Agency's avian pesticide assessment model. In RJ Kendall and TE Lacher, eds. *Wildlife Toxicology and Population Modeling: Integrated Studies of Agroecosystems. Proceedings of the Ninth Pellston Workshop*. CRC Press, Inc. Boca Raton, FA, pp 519-530.

Fitzpatrick D, Corish J, and Hayes B. 2004. Modelling skin permeability in risk assessment – the future. *Chemosphere* 55: 1309-1314.

Fleischli MA, Franson JC, Thomas NJ, Finley DL, and Riley Jr. W. 2004. Avian mortality events in the United States caused by anticholinesterase pesticides: a retrospective summary of National Wildlife Health Center records from 1980 to 2000. *Archives of Environmental Contamination and Toxicology* 46: 542-550.

Fleming WJ and Bradbury SP. 1981. Recovery of cholinesterase activity mallard ducklings administered organophosphorus pesticides. *Journal of Toxicology and Environmental Health* 8: 885-897.

Fleming WJ, Heinz GH, Franson JC, and Rattner BA. 1985. Toxicity of Abate® 4E (temephos) in mallard ducklings and the influence of cold. *Environmental Toxicology and Chemistry* 4: 193-199.

Franz TJ. 1983. Kinetics of cutaneous drug penetration. *International Journal of Dermatology* 22: 499-505.

Fossi MC, Leonzio C, Massi A, Lari L, and Casini S. 1992. Serum esterase inhibition in birds: a nondestructive biomarker to assess organophosphorus and carbamate contamination. *Archives of Environmental Contamination and Toxicology* 23: 99-104.

Fukuto TR. 1990. Mechanism of action of organophosphorous and carbamate insecticides. *Environmental Health Perspectives* 87: 245-254.

Gard NW and Hooper MJ. 1993. Age-dependent changes in plasma and brain cholinesterase activities of eastern bluebirds and European starlings. *Journal of Wildlife Diseases* 29: 1-7.

Geinoz S, Guy RH, Testa B, and Carrupt P.-A. 2004. Quantitative structure-permeation relationships (QSPeRs) to predict skin permeation: a critical evaluation. *Pharmaceutical Research* 21: 83-92.

Georgopolous PG, Sasso AF, Isukapalli SS, Lioy PJ, Vallero DA, Okino M, and Reiter L. 2009. Reconstructing population exposures to environmental chemicals from biomarkers: challenges and opportunities. *Journal of Exposure Science and Environmental Epidemiology* 19: 149-171.

Godin B and Touitou E. 2007. Transdermal skin delivery: predictions for humans from *in vivo, ex vivo* and animal models. *Advanced Drug Delivery Reviews* 59: 1152-1161.

Gordon CJ. 1996. Thermoregulatory aspects of environmental exposure to anticholinesterase agents. *Reviews on Environmental Health* 11: 101-117.

Gordon CJ and Padnos BK. 2000. Prolonged elevation in blood pressure in the unrestrained rat exposed to chlorpyrifos. *Toxicology* 146: 1-13.

Greig-Smith PW. 1994. Understanding the impact of pesticides on wild birds by monitoring incidents of poisoning. In RJ Kendall and TE Lacher, eds. *Wildlife Toxicology and Population Modeling: Integrated Studies of Agroecosystems. Proceedings of the Ninth Pellston Workshop*. CRC Press, Inc. Boca Raton, FA, pp 301-319.

Grue CE, Gibert PL, and Seeley ME. 1997. Neuophysiological and behavioral changes in non-target wildlife exposed to organophosphate and carbamate pesticides: thermoregulation, food consumption, and reproduction. *American Zoologist* 37: 369-388.

Grue CE, Hart ADM, and Mineau P. 1991. Biological consequences of depressed brain cholinesterase activity in wildlife. In P. Mineau, ed. *Cholinesterase-inhibiting insecticides: Their impact on wildlife and the environment*. Elsevier Science Publishing Company Inc., New York, NY, pp 151-209.

Grue CE and Hunter CC. 1984. Brain cholinesterase activity in fledgling starlings: implications for monitoring exposure of songbirds to ChE inhibitors. *Bulletin of Environmental Contamination and Toxicology* 32: 282-289.

Guy RH. 1996. Current status and future prospects of transdermal drug delivery. *Pharmaceutical Research* 13: 1765-1769.

Guy RH and Hadgraft J. 1988. Physicochemical aspects of percutaneous penetration and its enhancement. *Pharmaceutical Research* 5: 753-758.

Guy RH, Hadgraft J, and Maibach HI. 1982. A pharmacokinetic model for percutaneous absorption. *International Journal of Pharmaceutics* 11: 119-129.

Guy RH, Hadgraft J, and Maibach HI. 1985. Percutaneous absorption in man: a kinetic approach. *Toxicology and Applied Pharmacology* 78: 123-129.

Hansen ME and Wilson BW. 1999. Oxime reactivation of RBC acetylcholinesterase for biomonitoring. Archives of Environmental Contamination and Toxicology 37: 283-289.

Heinz GH. 1989. How lethal are sublethal effects? *Environmental Toxicology and Chemistry* 8: 463-464.

Henderson JD, Yamamoto JT, Fry DM, Seiber JN, and Wilson BW. 1994. Oral and dermal toxicity of organophosphate pesticides in the domestic pigeon (*Columba livia*). *Bulletin of Environmental Contamination and Toxicology* 52: 633-640.

Herin RA, Suggs JE, Lores EM, Heiderscheit LT, Farmer JD, and Pritsos C. 1978. Correlation of salt gland function with levels of chlorpyrifos in the feed of mallard ducklings. *Pesticide Biochemistry and Physiology* 9: 157-164.

Hill EF. 1989. Sex and storage affect cholinesterase activity in blood plasma of Japanese quail. *Journal of Wildlife Diseases* 25: 580-585.

Hill EF and Murray HC. 1987. Seasonal variation in diagnostic enzymes and biochemical constituents of captive northern bobwhites and passerines. *Comparative Biochemistry and Pharmacology* 87B: 933-940.

Hinz RS, Lorence CR, Hodson CD, Hansch C, Hall LL, and Guy RH. 1991. Percutaneous penetration of *para*-substituted phenols *in vitro*. *Fundamental and Applied Toxicology* 17: 575-583.

Hocking RR. 1996. Regression on functions of one variable. In Barnett V, Bradley RA, Fisher NI, Hunter JS, Kadane JB, Kendall DG, Scott DW, Smith AFM, Teugels JL, Watson GS, eds., *Methods and Applications of Linear Models: Regression and the Analysis of Variance*, John Wiley & Sons, Inc., New York, New York, USA, pp 131-182.

Hoerger FD and Kenaga EE. 1972. Pesticide residues on plants: correlation of representative data as a basis for estimation of their magnitude in the environment. In Coulston F and Korte F, eds. *Environmental Quality and Safety: Chemistry, Toxicology, and Technology, Vol. 1*, Academic Press, New York, NY, pp 9-28.

Hudson RH, Haegele MA, and Tucker RK. 1979. Acute oral and percutaneous toxicity of pesticides to mallards: correlations with mammalian toxicity data. *Toxicology and Applied Pharmacology* 47: 451-460.

Johns BE, Thompson RD, and Crane KA. 1992. Improved aerosol methodology for applying CPT to control roosting species of birds. *Proceedings of the Fifteenth Vertebrate Pest Conference*. Paper 41. University of Nebraska, Lincoln, Nebraska, pp 67-69.

Joyner PH, Kelly S, Shreve AA, Snead SE, Sleeman JM, and Pettit DA. 2006. West Nile virus in raptors from Virginia during 2003: clinical, diagnostic, and epidemiological findings. *Journal of Wildlife Diseases* 42: 335-344.

Kapustka L. 2008. Limitations of current practices used to perform ecological risk assessment. *Integrated Environmental Assessment and Management* 4: 290-298.

Kenaga EE. 1973. Factors to be considered in the evaluation of the toxicity of pesticides to birds in their environment. In *Environmental Quality and Safety*, Vol. 2. Academic Press, New York, NY, pp. 166-181.

Kramer RE, Wellman SE, Rockhold RW, and Baker RC. 2002a. Pharmacokinetics of methyl parathion: a comparison following single intravenous, oral or dermal administration. *Journal of Biomedical Sciences* 9: 311-320.

Kramer RE, Wellman SE, Zhu H, Rockhold RW, and Baker RC. 2002b. A comparison of cholinesterase activity after intravenous, oral or dermal administration of methyl parathion. *Journal of Biomedical Sciences* 9: 140-148.

Lane ME, Santos P, Watkinson AC, and Hadgraft J. 2012. Passive skin permeation enhancement. In Benson HAE, Watkinson AC, eds, *Topical and transdermal drug delivery: principles and practice*, 1st edition, John Wiley & Sons, Inc., Hoboken, New Jersey, USA, pp 23-42.

Levine TR and Hullett CR. 2002. Eta squared, partial eta squared, and misreporting of effect size in communication research. *Human Communication Research* 28: 612-625.

Lian G, Chen L, and Han L. 2008. An evaluation of mathematical models for predicting skin permeability. *Journal of Pharmaceutical Sciences* 97: 584-598.

Lindena J, Kupper W, and Trautschold I. 1986. Catalytic enzyme activity concentration in thoracic duct, liver, and intestinal lymph of the dog, the rabbit, the rat and the mouse. *Journal of Clinical Chemistry and Clinical Biochemistry* 24: 19-33.

Lipinski CA, Lombardo F, Dominy BW, and Feeney PJ. 2001. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Advanced Drug Delivery Reviews* 46: 3-26.

Ludke JL, Hill EF, and Dieter MP. 1975. Cholinesterase (ChE) response and related mortality among birds fed ChE inhibitors. *Archives of Environmental Contamination and Toxicology* 3: 1-21.

MacLachlan DJ. 2010. Physiologically based pharmacokinetic (PBPK) model for residues of lipophilic pesticides in poultry. *Food Additives and Contaminants* 27: 302-314.

Maguire CC and Williams BA. 1987. Response of thermal stressed bobwhite to organophosphorous exposure. *Environmental Pollution* 47: 25-39.

Makin A and Mortensen JT. 2012. Toxicological and pre-clinical considerations for novel excipients and new chemical entities. In Benson HAE, Watkinson AC, eds., *Topical and Transdermal Drug Delivery: Principles and Practice, 1st ed.*, John Wiley & Sons, Inc., Hoboken, New Jersey, USA, pp 233-254.

Martin PA and Solomon KR. 1991. Acute carbofuran exposure and cold stress: interactive effects in mallard ducklings. *Pesticide Biochemistry and Physiology* 40: 117-127.

Matz AC, Bennett RS, and Landis WG. 1998. Effects of azinphos-methyl on northern bobwhite: a comparison of laboratory and field results. *Environmental Toxicology and Chemistry* 17: 1364-1370.

Maul JD and Farris JL. 2004. The effect of sex on avian plasma cholinesterase enzyme activity: a potential source of variation in an avian biomarker endpoint. *Archives of Environmental Contamination and Toxicology* 47: 253-258.

Maul JD and Farris JL. 2005. Monitoring exposure of Northern cardinals, *Cardinalis cardinalis*, to cholinesterase-inhibiting pesticides: enzyme activity, reactivations, and indicators of environmental stress. *Environmental Toxicology and Chemistry* 24: 1721-1730.

McEwen LC, DeWeese LR, and Schladweiler P. 1986. Bird predation on cutworms (Lepidoptera: Noctuidae) in wheat fields and chlorpyrifos effects on brain cholinesterase activity. *Environmental Entomology* 15: 147-151.

McFarland LZ and Lacy PB. 1968. Acute anticholinesterase toxicity in ducks and Japanese quail. *Toxicol Appl Pharmacol* 12: 105-114.

McInnes PF, Andersen DE, Hoff DJ, Hooper MJ, and Kinkel LL. 1996. Monitoring exposure of nestling songbirds to agricultural application of an organophosphorus insecticide using cholinesterase activity. *Environmental Toxicology and Chemistry* 15: 544-552.

Meteyer CU, Dubielzig RR, Dein FJ, Baeten LA, Moore MIS, Jehl Jr. JR, and Wesenberg K. 1997. Sodium toxicity and pathology associated with exposure of waterfowl to hypersaline playa lakes of southeast New Mexico. *J Vet Diagn Invest* 9: 269-280.

Miller KJ. 2012. Transdermal product formulation development. In Benson HAE, Watkinson AC, eds., *Topical and Transdermal Drug Delivery: Principles and Practice, 1st ed.*, John Wiley & Sons, Inc., Hoboken, New Jersey, USA, pp 287-308.

Mineau P. 1991. Difficulties in the regulatory assessment of cholinesterase-inhibiting insecticides. In Mineau P, ed., *Chemicals in Agriculture, Volume 2 – Cholinesterase-inhibiting Insecticides, Their Impact on Wildlife and the Environment*, Elsevier, Amsterdam, The Netherlands, pp 277-299.

Mineau P. 2002. Estimating the probability of bird mortality from pesticide sprays on the basis of the field study record. *Environmental Toxicology and Chemistry* 21: 1497-1506.

Mineau P. 2005. Direct losses of birds to pesticides – beginnings of a quantification. In CJ Ralph and TD Rich, eds. *Bird Conservation Implementation and Integration in the Americas: Third International Partners in Flight Conference 2002, Vol. 2*, U.S. Department of Agriculture, Albany, CA. pp 1065-1070.

Mineau P. 2007. Developing risk-based rankings for pesticides in support of standard development at Environment Canada: Risk-based approach for terrestrial biota continued –

incorporating dermal exposure in pesticide risk assessment for birds. National Agri-Environmental Standards Initiative Technical Series Report No. 3-32.

Mineau P, Sundaram KMS, Sundaram S, Feng C, Busby DG, and Pearce PA. 1990. An improved method to study the impact of pesticide sprays on small song birds. *Journal of Environmental Science and Health, Part B* 25: 105-135.

Mineau P and Whiteside M. 2006. Lethal risk to birds from insecticide use in the United States – a spatial and temporal analysis. *Environmental Toxicology and Chemistry* 25: 1214-1222.

Miselnicky SR, Lichtin JL, Sakr A, and Bronaugh RL. 1988. The influence of solubility, protein binding, and percutaneous absorption on reservoir formation in skin. *Journal of the Society of Cosmetic Chemists* 39: 169-177.

Mitragotri S. 2002. A theoretical analysis of permeation of small hydrophobic solutes across the stratum corneum based on scaled particle theory. *Journal of Pharmaceutical Sciences* 91: 744-752.

Mosquin PL, Licata AC, Liu B, Sumner SCJ, and Okino MS. 2009. Reconstructing exposures from small samples using physiologically based pharmacokinetic models and multiple biomarkers. *Journal of Exposure Science and Environmental Epidemiology* 19: 284-297.

Moss GP and Cronin MTD. 2002. Quantitative structure-permeability relationships for percutaneous absorption: re-analysis of steroid data. *International Journal of Pharmaceutics* 238: 105-109.

Moss GP, Dearden JC, Patel H, and Cronin MTD. 2002. Quantitative structure-permeability relationships (QSRPs) for percutaneous absorption. *Toxicology in Vitro* 16: 299-317.

Neimanis A, Gavier-Widen D, Leighton F, Bollinger T, Rocke T, and Morner T. 2007. An outbreak of type C botulism in herring gulls (*Larus argentatus*) in southwestern Sweden. *Journal of Wildlife Diseases* 43: 327-336.

Niethammer KR and Baskett TS. 1983. Cholinesterase inhibition of birds inhabiting wheat fields treated with methyl parathion and toxaphene. *Archives of Environmental Contamination and Toxicology* 12: 471-475.

Nose H, Morimoto T, and Ogura K. 1983. Distribution of water losses among fluid compartments of tissues under thermal dehydration in the rat. *Japanese Journal of Physiology* 33: 1019-1029.

OECD. 2004a. Skin absorption: *In vivo* method. OECD Guidelines for the Testing of Chemicals. Test No. 427. OECD. Paris, France.

OECD. 2004b. Skin absorption: *In vitro* method. OECD Guidelines for the Testing of Chemicals. Test No. 428. OECD. Paris, France.

Olanrewaju HA, Thaxton JP, Dozier WA, and Branton SL. 2007. Electrolyte diets, stress, and acid-base balance in broiler chickens. *Poultry Science* 86: 1363-1371.

Pagliara A, Reist M, Geinoz S, Carrupt P-A, and Testa B. 1999. Evaluation and prediction of drug permeation. *Journal of Pharmacy and Pharmacology* 51: 1339-1357.

Pass DA. 1995. Normal anatomy of the avian skin and feathers. *Seminars in Avian and Exotic Pet Medicine* 4: 152-160.

Pearson K. 1911. On a correction needful in the case of the correlation ratio. *Biometrika* 8: 254-256.

Pollet RA, Glatz CE, and Dyer DC. 1985. The pharmacokinetics of chlortetracycline orally administered to turkeys: influence of citric acid and *Pasteurella multocida* infection. *Journal of Pharmacokinetics and Biopharmaceutics* 13: 243-264.

Pope C, Karanth S, and Liu J. 2005. Pharmacology and toxicology of cholinesterase inhibitors: uses and misuses of a common mechanism of action. *Environmental Toxicology and Pharmacology* 19: 433-446.

Pope GG and Ward P. 1972. The effects of small applications of an organophosphorus poison, fenthion, on the weaver-bird *Quelea quelea*. *Toxicology and Applied Pharmacology* 3: 197-205.

Potts RO and Guy RH. 1992. Predicting skin permeability. *Pharmaceutical Research* 9: 663-669.

Power M and Adams SM. 1997. Charlatan or sage? A dichotomy of views on ecological risk assessment. *Environmental Management* 21: 803-830.

Prijono WB and Leighton FA. 1991. Parallel measurements of brain acetylcholinesterase and the muscarinic cholinergic receptor in the diagnosis of acute, lethal poisoning by anti-cholinesterase pesticides. *Journal of Wildlife Diseases* 27: 110-115.

Qiao GL, Williams PL, and Riviere JE. 1994. Percutaneous absorption, biotransformation, and systemic disposition of parathion in vivo in swine. I. Comprehensive pharmacokinetic model. *Drug Metabolism and Disposition* 22: 459-471.

Rattner BA. 1982. Diagnosis of anticholinesterase poisoning in birds: effects of environmental temperature and underfeeding on cholinesterase activity. *Environmental Toxicology and Chemistry* 1: 329-335.

Rattner BA, Becker JM, and Nakatsugawa T. 1987. Enhancement of parathion toxicity to quail by heat and cold exposure. *Pesticide Biochemistry and Physiology* 27: 330-339.

Rattner BA and Fairbrother A. 1991. Biological variability and the influence of stress on cholinesterase activity. In P Mineau, ed. *Cholinesterase-inhibiting insecticides: their impact on wildlife and the environment*. Elsevier Science Publishing Company Inc., New York, NY, pp 89-107.

Rattner BA, Fleming WJ, and Murray HC. 1983. Osmoregulatory function in ducks following ingestion of the organophosphorus insecticide fenthion. *Pesticide Biochemistry and Physiology* 20: 246-255.

Rattner BA and Franson JC. 1984. Methyl parathion and fenvalerate toxicity in American kestrels: acute physiological responses and effects of cold. *Canadian Journal of Physiology and Pharmacology* 62: 787-792.

Rendon-von Osten J, Soares AMVM, and Guilherminos L. 2005. Black-bellied whistling duck (*Dendrocygna autumnalis*) brain cholinesterase characterization and diagnosis of anticholinesterase pesticide exposure in wild populations from Mexico. *Environmental Toxicology and Chemistry* 24: 313-317.

Roberts JR and Dantzler WH. 1989. Glomerular filtration rate in conscious unrestrained starlings under dehydration. *American Journal of Physiology* 256: R836-R839.

Roberts MS, Cross SE, and Anissimov YG. 2004. Factors affecting the formation of a skin reservoir for topically applied solutes. *Skin Pharmacology and Physiology* 17: 3-16.

Robinson SC, Kendall RJ, Robinson R, Driver CJ, and Lacher, Jr. TE. 1988. Effects of agricultural spraying of methyl parathion on cholinesterase activity and reproductive success in wild starlings (*Sturnus vulgaris*). *Environmental Toxicology and Chemistry* 7: 343-349.

Rogers Jr. JG, Cagan RH, and Kare MR. 1974. Percutaneous absorption of several chemicals, pesticides included, in the red-winged blackbird. *Environmental Physiology and Biochemistry* 4: 104-111.

Rougier A. 2005. In vivo percutaneous absorption: a key role for stratum corneum/vehicle partitioning. In Bronaugh RL, Maibach HI, eds, *Percutaneous Absorption: Drugs-Cosmetics-Mechanisms-Methodology, 4th ed.*, Taylor & Francis Group, LLC, Boca Raton, Florida, USA, pp 123-137.

Sartorelli P, Aprea C, Cenni A, Novelli, MT, Orsi D, Palmi S, and Matteucci G. 1998. Prediction of percutaneous absorption from physicochemical data: a model based on data of *in vitro* experiments. *Annals of Occupational Hygiene* 42: 267-276.

Schafer Jr. EW. 1984. Potential primary and secondary hazards of avicides. *Proceedings of the Eleventh Vertebrate Pest Conference*. Paper 35. University of Nebraska, Lincoln, Nebraska, pp 217-222.

Schafer Jr. EW, Brunton RB, Lockyer NF, and De Grazio JW. 1973. Comparative toxicity of seventeen pesticides to the Quelea, house sparrow, and red-winged blackbird. *Toxicology and Applied Pharmacology* 26: 154-157.

Schleuplein RJ and Ross LW. 1974. Mechanism of percutaneous absorption. V. Percutaneous absorption of solvent deposited solids. *The Journal of Investigative Dermatology* 62: 353-360.

Schwab R. 1968. Roost treatments using 3-chloro *p*-toluidine hydrochloride. *Bird Control Seminars Proceedings*. Paper 173. University of Nebraska, Lincoln, Nebraska, pp 136-139.

Shumake SA and Savarie PJ. 1996. Nest material as a delivery method for avicides: preliminary tests with African weaver finches. *Proceedings of the Seventeenth Vertebrate Pest Conference*. University of California, Davis, California, pp 113-118.

Smith EG and Gordon CJ. 2005. The effects of chlorpyrifos on blood pressure and temperature regulation in spontaneously hypertensive rats. *Basic & Clinical Pharmacology & Toxicology* 96: 503-511.

Smith MR, Thomas NJ, and Hulse C. 1995. Application of brain cholinesterase reactivation to differentiate between organophosphorus and carbamate pesticide exposure in wild birds. *Journal of Wildlife Diseases* 31: 263-267.

Soler-Rodriguez F, Miguez-Santiyan MP, Reja-Sanchez A, Roncero-Cordero V, and Garcia-Cambero JP. 1998. Recovery of brain acetylcholinesterase and plasma cholinesterase activities in quail (*Coturnix coturnix*) after chlorpyriphos administration and effect of pralidoxime treatment. *Environmental Toxicology and Chemistry* 17: 1835-1839.

Stansley W. 1993. Field results using cholinesterase reactivation techniques to diagnose acute anticholinesterase poisoning in birds and fish. *Archives of Environmental Contamination and Toxicology* 25: 315-321.

Subedi KS, Oh SY, Chun M-K, and Choi H-K. 2010. Recent advances in transdermal drug delivery. *Archives of Pharmaceutical Research* 33: 339-351.

Suter II GW. 2008. Ecological risk assessment in the United States Environmental Protection Agency: a historical overview. *Integrated Environmental Assessment and Management* 4: 285-289.

Takei Y, Okawara Y, and Kobayashi H. 1988. Water intake induced by water deprivation in the quail, *Coturnix coturnix japonica*. *Journal of Comparative Physiology B* 158: 519-525.

Tan Y.-M., Liao KH, Conolly RB, Blount BC, Mason AM, and Clewell HJ. 2006. Use of a physiologically based pharmacokinetic model to identify exposures consistent with human biomonitoring data for chloroform. *Journal of Toxicology and Environmental Health Part A* 69: 1727-1756.

Terasawa S, Itsuki H, and Arakawa S. 1975. Contributions of hydrogen bonds to the partial molar volumes of nonionic solutes in water. *Journal of Physical Chemistry* 79: 2345-2351.

Thompson HM. 1991. Serum "B" esterases as indicators of exposure to pesticides. In P Mineau, ed. *Cholinesterase-inhibiting insecticides: their impact on wildlife and the environment*. Elsevier Science Publishing Company Inc., New York, NY, pp 109-125.

Thompson HM. 1999. Esterases as markers of exposure to organophosphates and carbamates. *Ecotoxicology* 8: 369-384.

Tice CM. 2001. Selecting the right compounds for screening: does Lipinski's Rule of 5 for pharmaceuticals apply to agrochemicals? *Pest Management Science* 57: 3-16.

Tiebout IHM and Brugger KE. 1995. Ecological risk assessment of pesticides for terrestrial vertebrates: evaluation and application of the U.S. Environmental Protection Agency's quotient method. *Conservation Biology* 9: 1605-1618.

Timchalk C, Nolan RJ, Mendrala AL, Dittenber DA, Brzak KA, and Mattsson JL. 2002. A physiologically based pharmacokinetic and pharmacodynamic (PBPK/PD) model for the organophosphate insecticide chlorpyrifos in rats and humans. *Toxicological Sciences* 66: 34-53.

Underwood H. 1994. The circadian rhythm of thermoregulation in Japanese quail: I. Role of the eyes and pineal. *Journal of Comparative Physiology A* 175: 639-653.

Urban DJ and Cook NJ. 1986. Ecological risk assessment. Standard evaluation procedure. EPA 540/9-85-001. U.S. Environmental Protection Agency, Washington, DC.

van de Sandt JJM, van Burgsteden JA, Cage S, Carmichael PL, Dick I, Kenyon S, Korinth G, Larese F, Limasset JC, Mass WJM, Montomoli L, Nielsen JB, Payan J-P, Robinson E, Sartorelli P, Schaller KH, Wilkinson SC, and Williams FM. 2004. *In vitro* predictions of skin absorption of caffeine, testosterone, and benzoic acid: a multi-center comparison study. *Regulatory Toxicology and Pharmacology* 39: 271-281.

van der Merwe D, Brooks JD, Gehring R, Baynes RE, Monteiro-Riviere NA, and Riviere JE. 2006. A physiologically based pharmacokinetic model of organophosphate dermal absorption. *Toxicological Sciences* 89: 188-204.

Vickers C. 1963. Existence of a reservoir in the stratum corneum: experimental proof. *Archives of Dermatology* 88: 72-75.

Vyas NB. 1999. Factors influencing estimation of pesticide-related wildlife mortality. *Toxicology and Industrial Health* 15: 187-192.

Vyas NB, Spann JW, Hulse CS, Borges SL, Bennett RS, Torrez M, Williams BI, and Leffel R. 2006. Field evaluation of an avian risk assessment model. *Environmental Toxicology and Chemistry* 25: 1762-1771.

Vyas NB, Spann JW, Hulse CS, Gentry S, and Borges SL. 2007. Dermal insecticide residues from birds inhabiting an orchard. *Environmental Monitoring and Assessment* 133: 209-214.

Vyas NB, Spann JW, Hulse CS, Torrez M, Williams BI, and Leffel R. 2004. Decomponsed gosling feet provide evidence of insecticide exposure. *Environmental Monitoring and Assessment* 98: 351-361.

Walker CH. 2003. Neurotoxic pesticides and behavioural effects upon birds. *Ecotoxicology* 12: 307-316.

Wang G, Edge W, and Wolff JO. 1999. A field test of the quotient method for predicting risk to *Microtus canicaudus* in grasslands. *Archives of Environmental Contamination and Toxicology* 36: 207-212.

Westlake GE, Bunyan PJ, Martin AD, Stanley PI, and Steed LC. 1981. Organophosphate poisoning. Effects of selected organophosphate pesticides on plasma enzymes and brain esterases of Japanese quail (*Coturnix coturnix japonica*). *Journal of Agriculture and Food Chemistry* 29: 772-778.

Windingstad RM, Kartch FX, Stroud RK, and Smith MR. 1987. Salt toxicosis in waterfowl in North Dakota. *Journal of Wildlife Diseases* 23: 443-446.

Wilson L, Martin PA, Elliott JE, Mineau P, and Cheng KM. 2001. Exposure of California quail to organophosphorus insecticides in apple orchards in the Okanagan Valley, British Columbia. *Ecotoxicology* 10: 79-90.

Wynne J and Stringfield C. 2007. Treatment of lead toxicity and crop stasis in a California condor (*Gymnogyps californianus*). *Journal of Zoo and Wildlife Medicine* 38: 588-590.

Yagi S, Nakayama K, Kurosaki K, and Kimura T. 1998. Factors determining drug residence in skin during transdermal absorption: studies on β-blocking agents. *Biological and Pharmaceutical Bulletin* 21: 1195-1201.

Yano T, Nakagawa A, Tsuji M, and Noda K. 1986. Skin permeability of various non-steroidal anti-inflammatory drugs in man. *Life Sciences* 39: 1043-1050.

Zar JH. 1999. *Biostatistical analysis*. Prentice Hall, Upper Saddle River, New Jersey, USA, 663 pp.

Zhang Q, Grice JE, Li P, Jepps OG, Wang G.-J., and Roberts MS. 2009. Skin solubility determines maximum transepidermal flux for similar size molecules. *Pharmaceutical Research* 26: 1974-1985.

APPENDIX A:

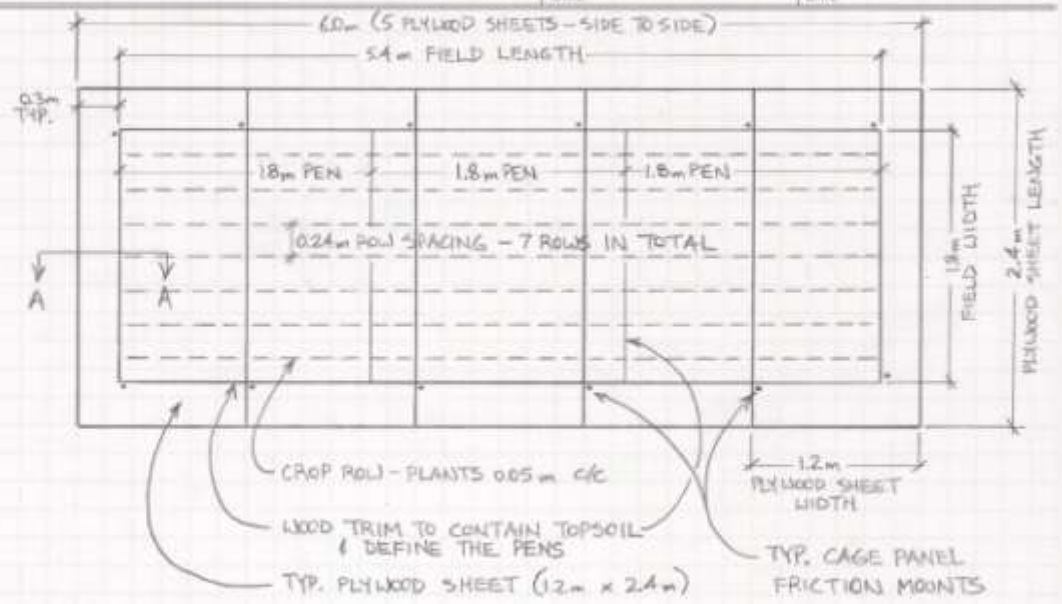
SIMULATED FIELD SCHEMATICS

PROBLEMS

CLASS

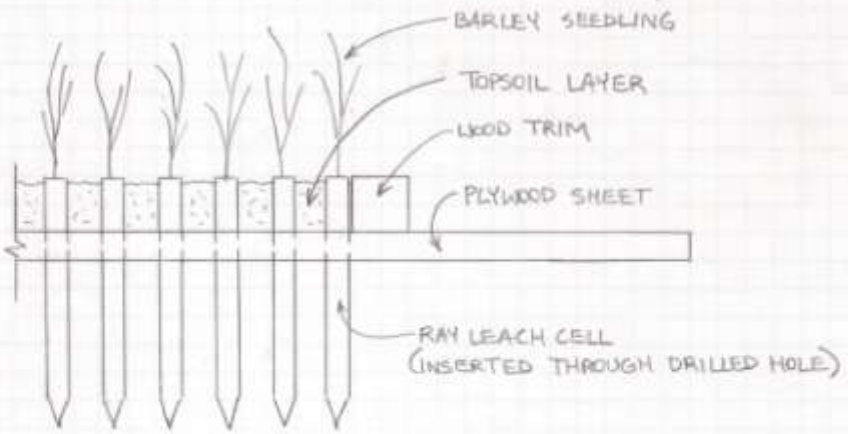
NAME

DATE



SIMULATED FIELD – PLAN VIEW

SCALE: APPROX. 40:1



SIMULATED FIELD – SECTION A-A

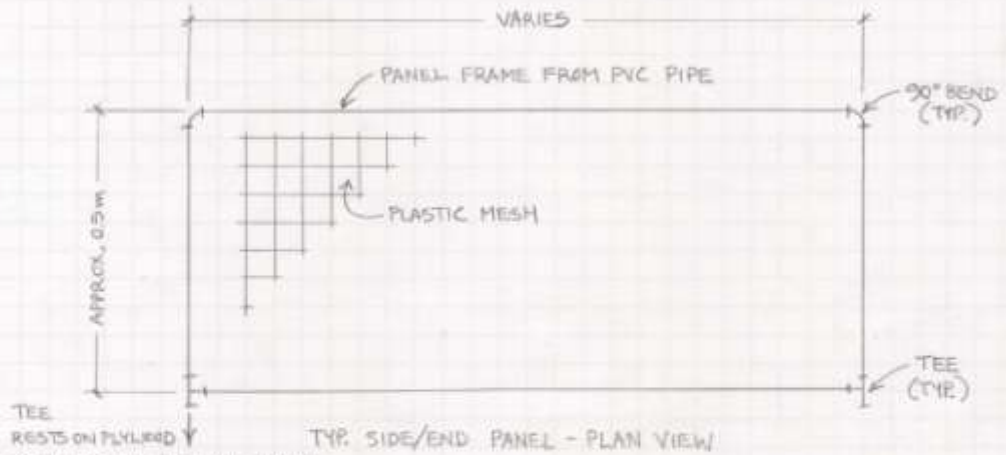
NOT TO SCALE

PROBLEMS

CLASS

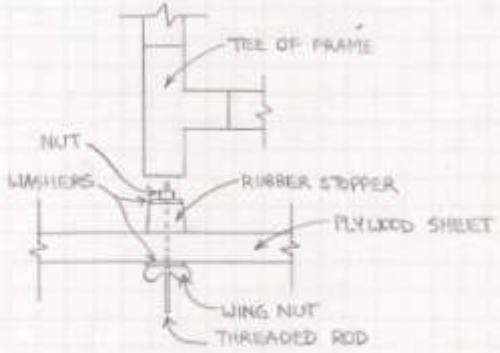
NAME

DATE



TYP. SIDE/END PANEL - PLAN VIEW

TYP. TOP PANEL - PLAN VIEW



FRICTION MOUNT DETAIL

NOTES:

1. ALL PIPE AND FITTINGS OF FRAME ARE 32 mm (1¼") DIAMETER PVC PIPE.
2. PLASTIC MESH IS ANCHORED TO FRAME WITH CABLE TIES.
3. PANELS ARE ANCHORED TO EACH OTHER DURING ASSEMBLY WITH CABLE TIES.

FIELD CAGE PANEL SCHEMATICS

NOT TO SCALE

APPENDIX B:

GRAPHICAL RECORD OF CHOLINESTERASE ASSAYS OF PRE-EXPOSURE AND POST-EXPOSURE PLASMA SAMPLES BY DATE

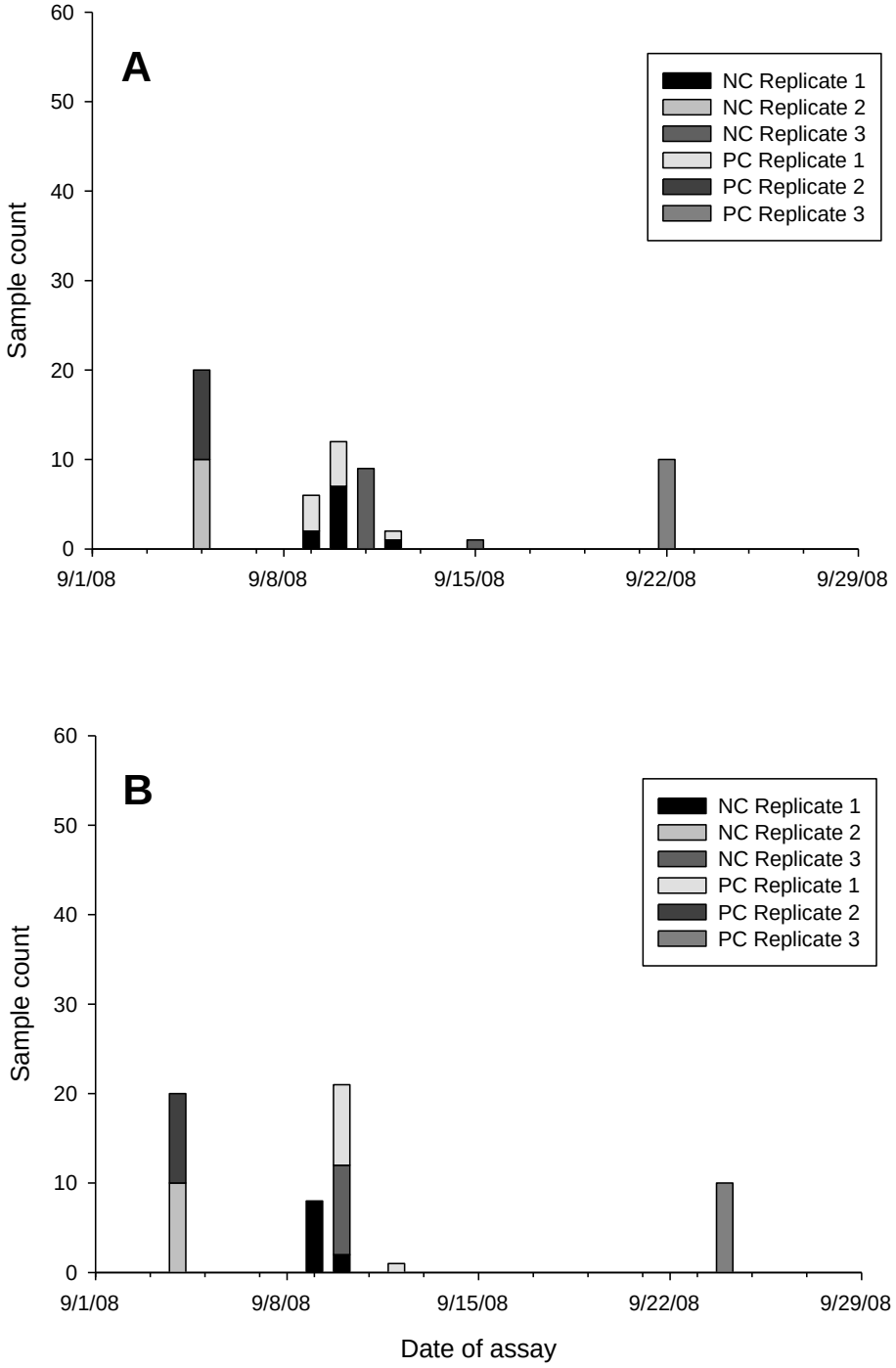


Fig. A1. Distribution of quail plasma samples from dicrotophos negative control (NC) and positive control (PC) exposures within cholinesterase assays by experimental replicate and date for both the pre-exposure (**A**) and post-exposure (**B**) sampling periods.

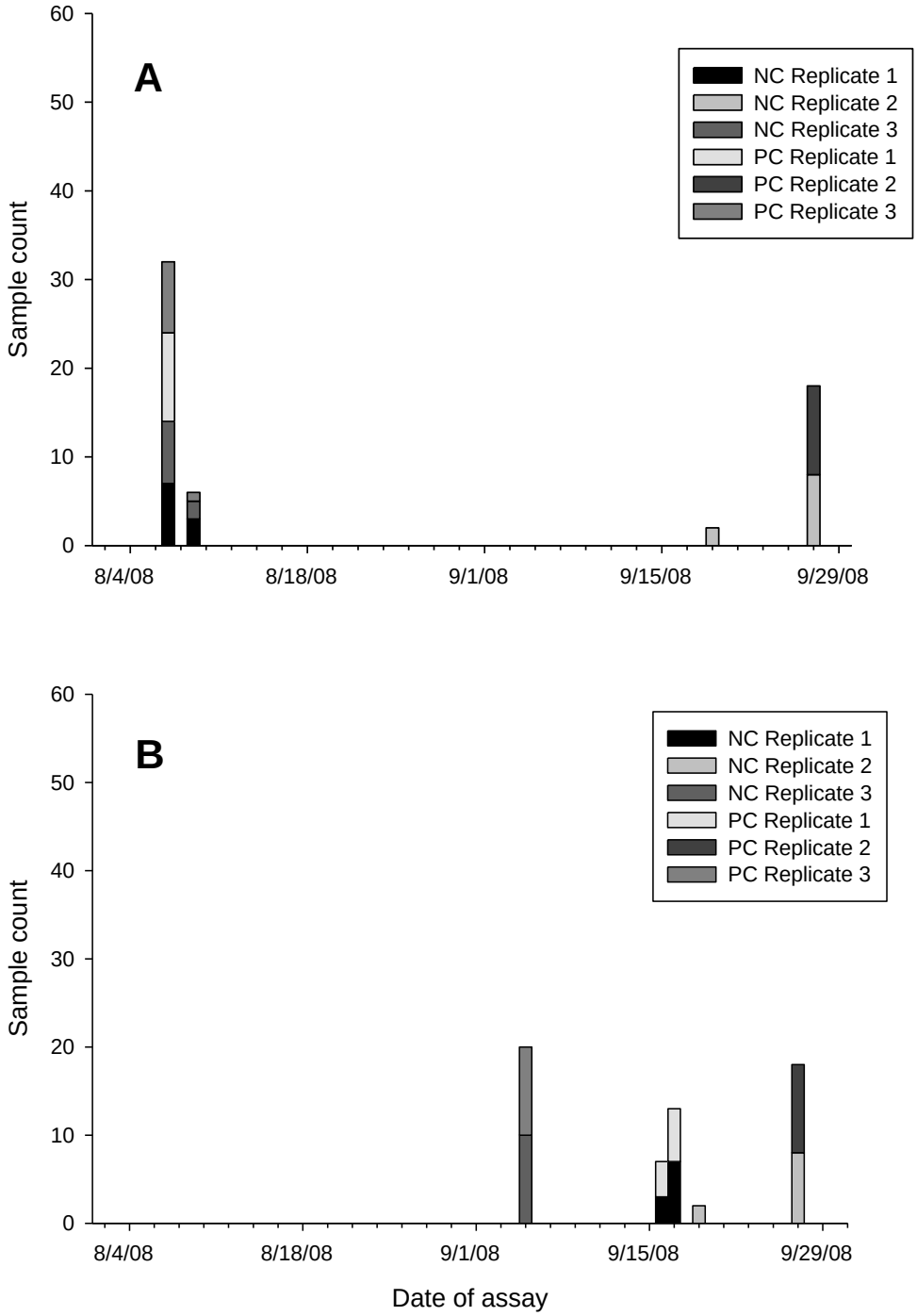


Fig. A2. Distribution of quail plasma samples from ethoprop negative control (NC) and positive control (PC) exposures within cholinesterase assays by experimental replicate and date for both the pre-exposure (**A**) and post-exposure (**B**) sampling periods.

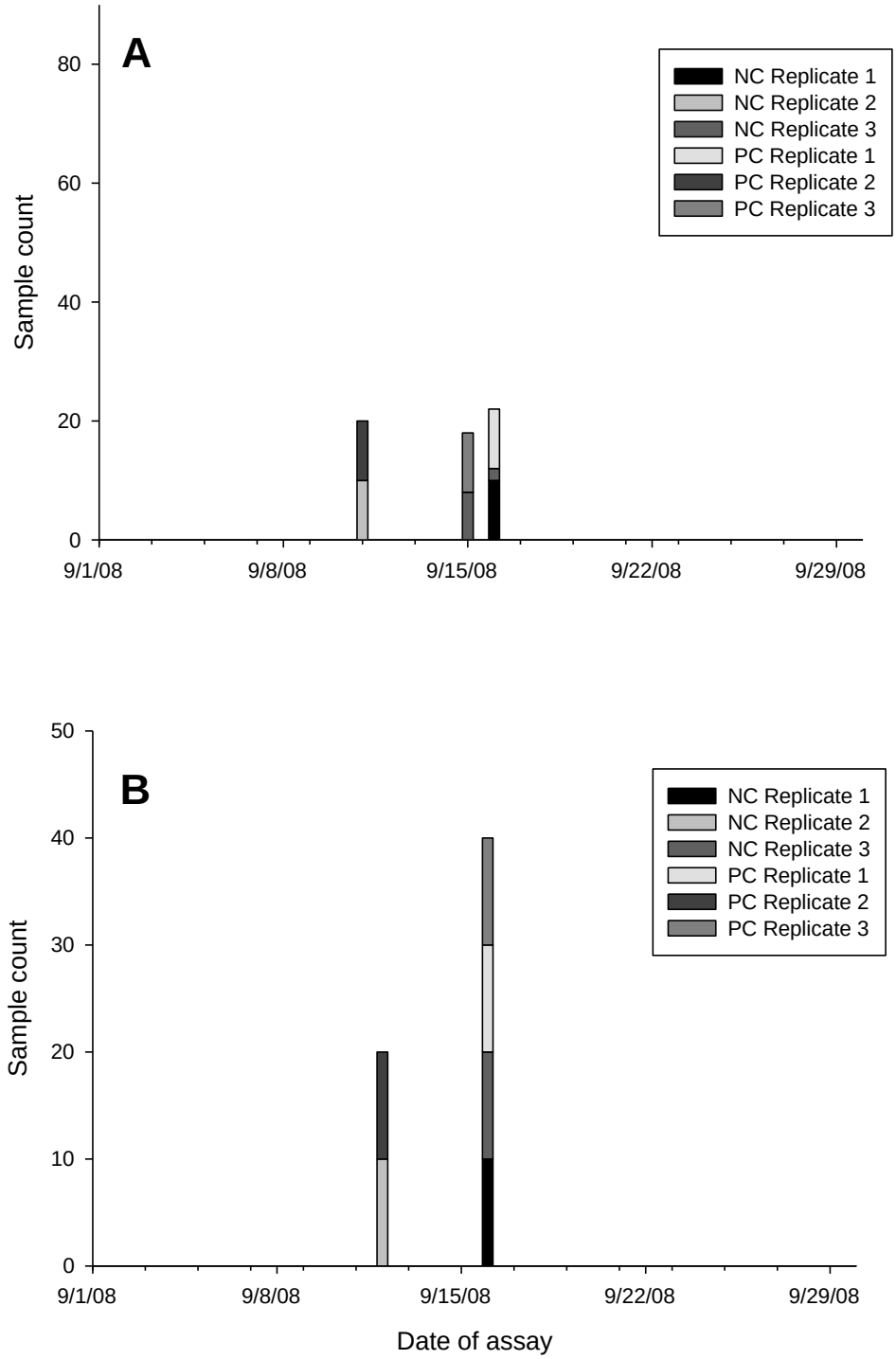


Fig. A3. Distribution of quail plasma samples from methamidophos negative control (NC) and positive control (PC) exposures within cholinesterase assays by experimental replicate and date for both the pre-exposure (**A**) and post-exposure (**B**) sampling periods.

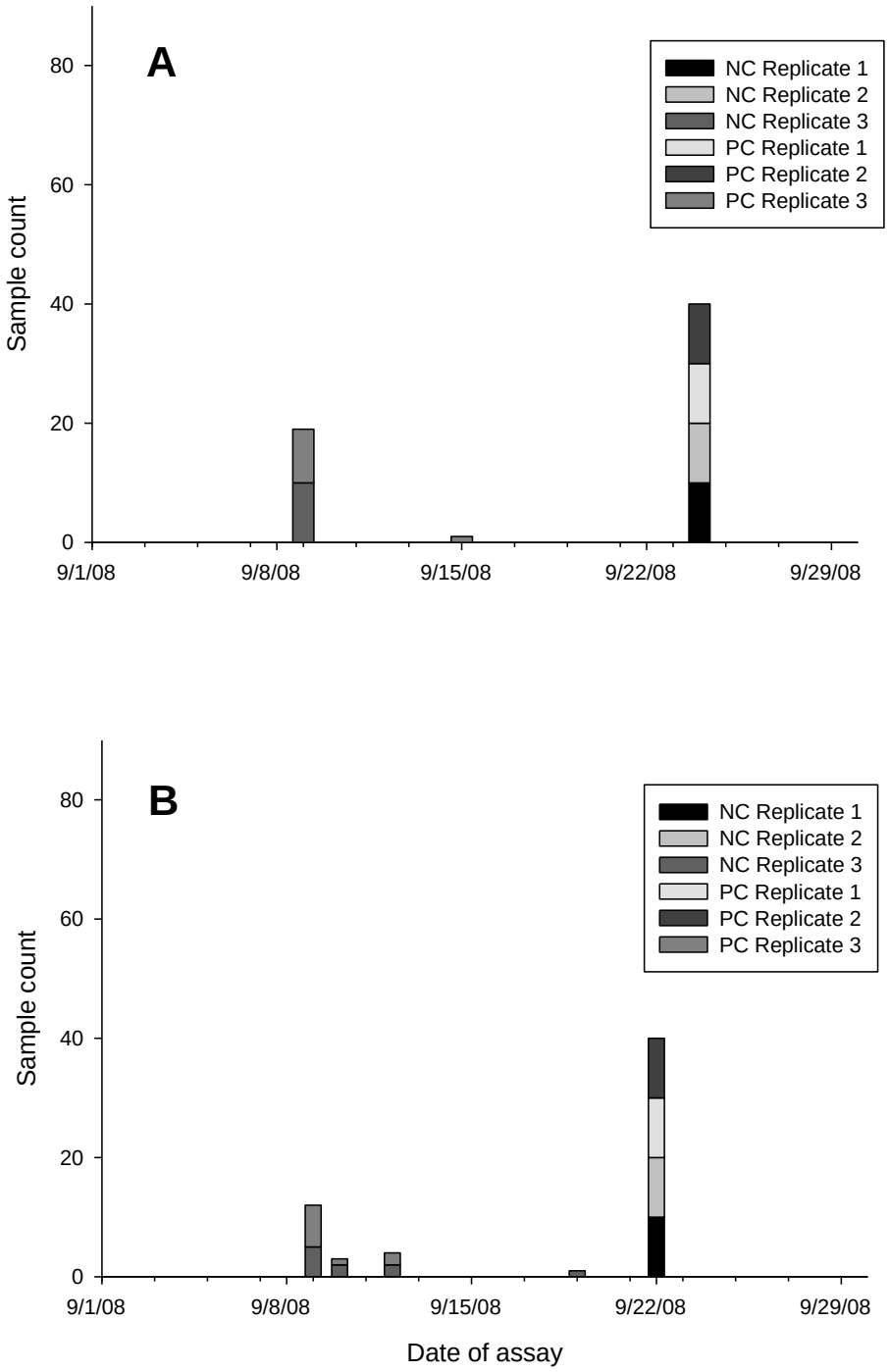


Fig. A4. Distribution of quail plasma samples from naled negative control (NC) and positive control (PC) exposures within cholinesterase assays by experimental replicate and date for both the pre-exposure (**A**) and post-exposure (**B**) sampling periods.

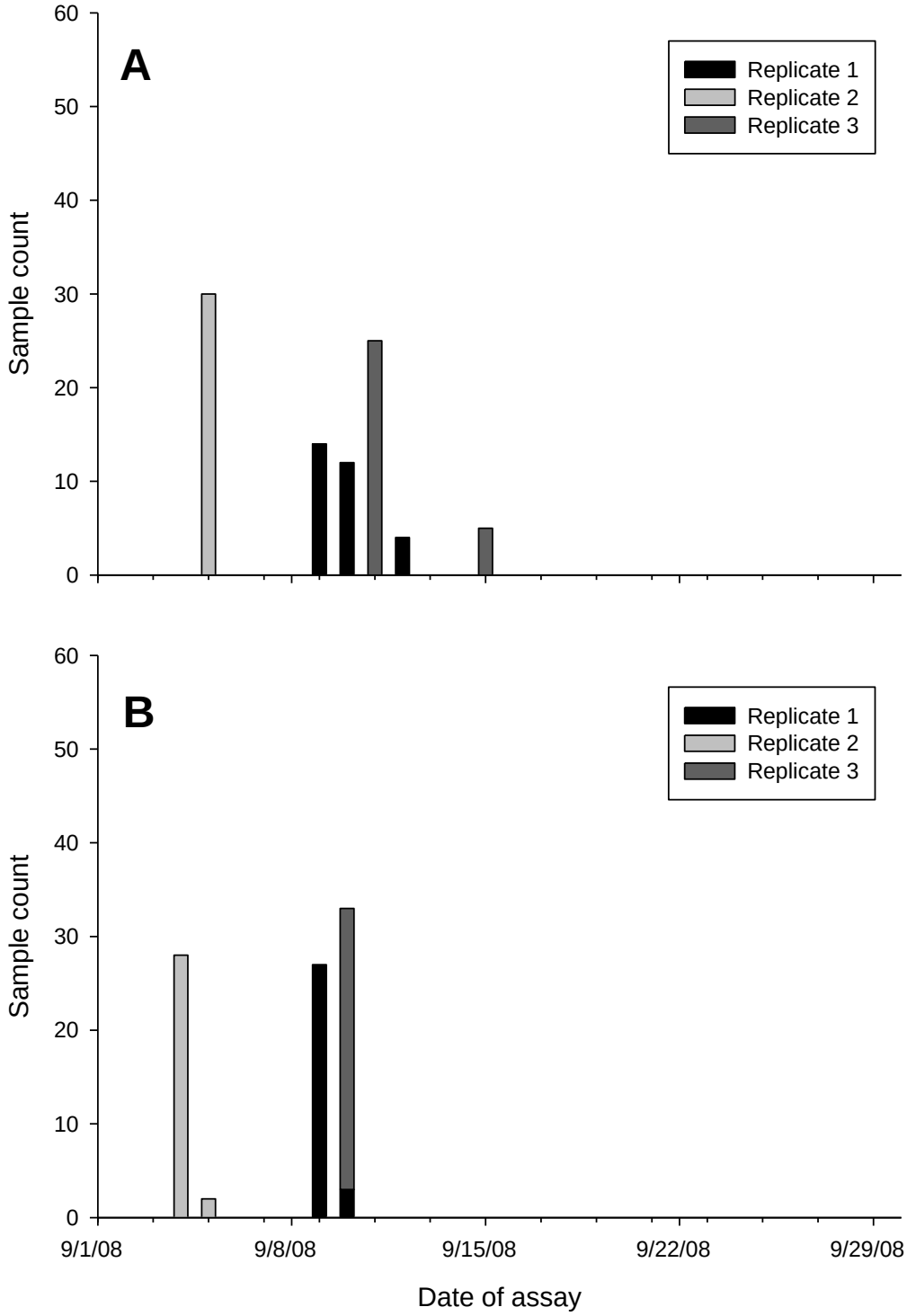


Fig. A5. Distribution of quail plasma samples from dicrotophos simulated field exposures within cholinesterase assays by experimental replicate and date for both the pre-exposure (**A**) and post-exposure (**B**) sampling periods.

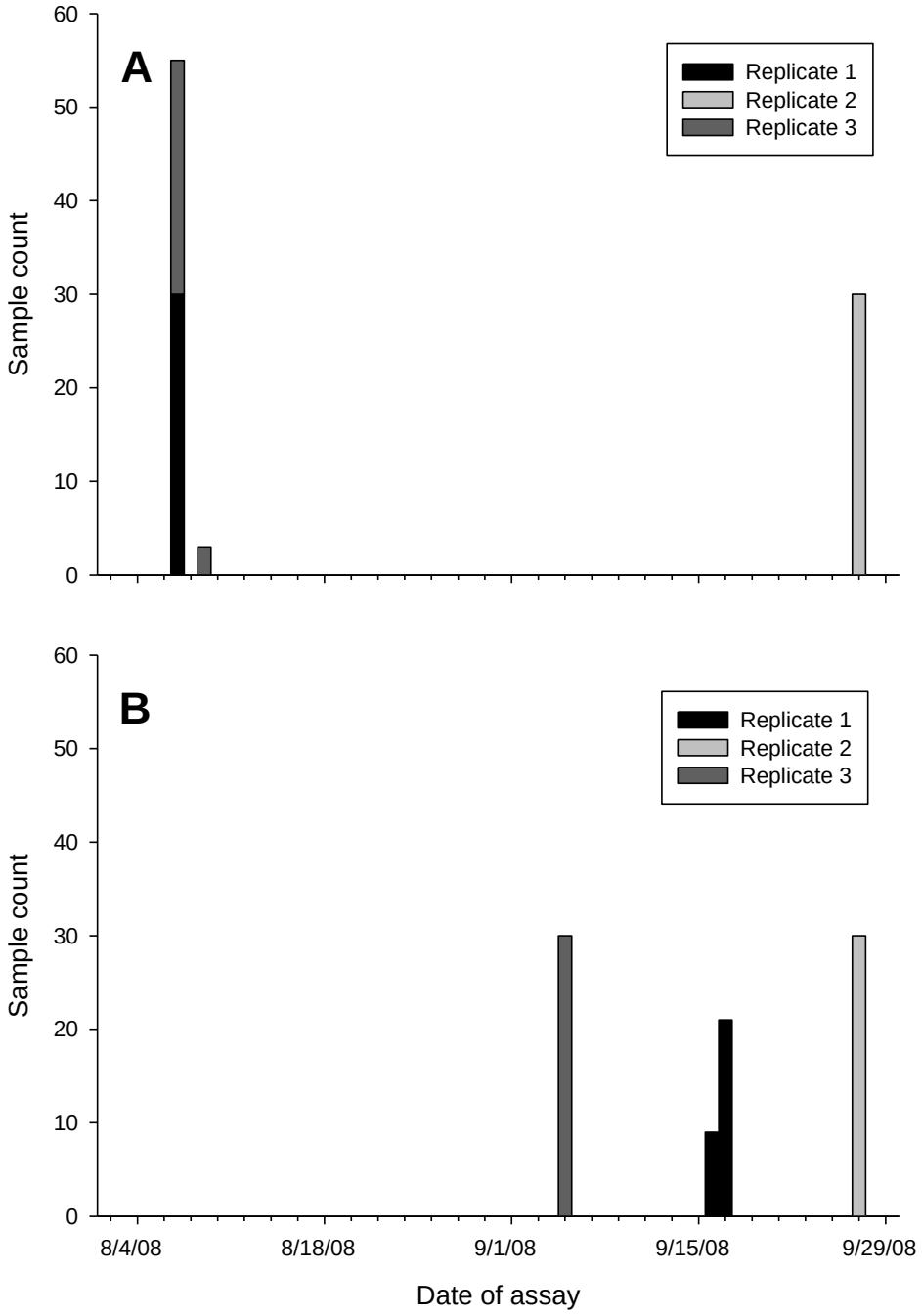


Fig. A6. Distribution of quail plasma samples from ethoprop simulated field exposures within cholinesterase assays by experimental replicate and date for both the pre-exposure (**A**) and post-exposure (**B**) sampling periods.

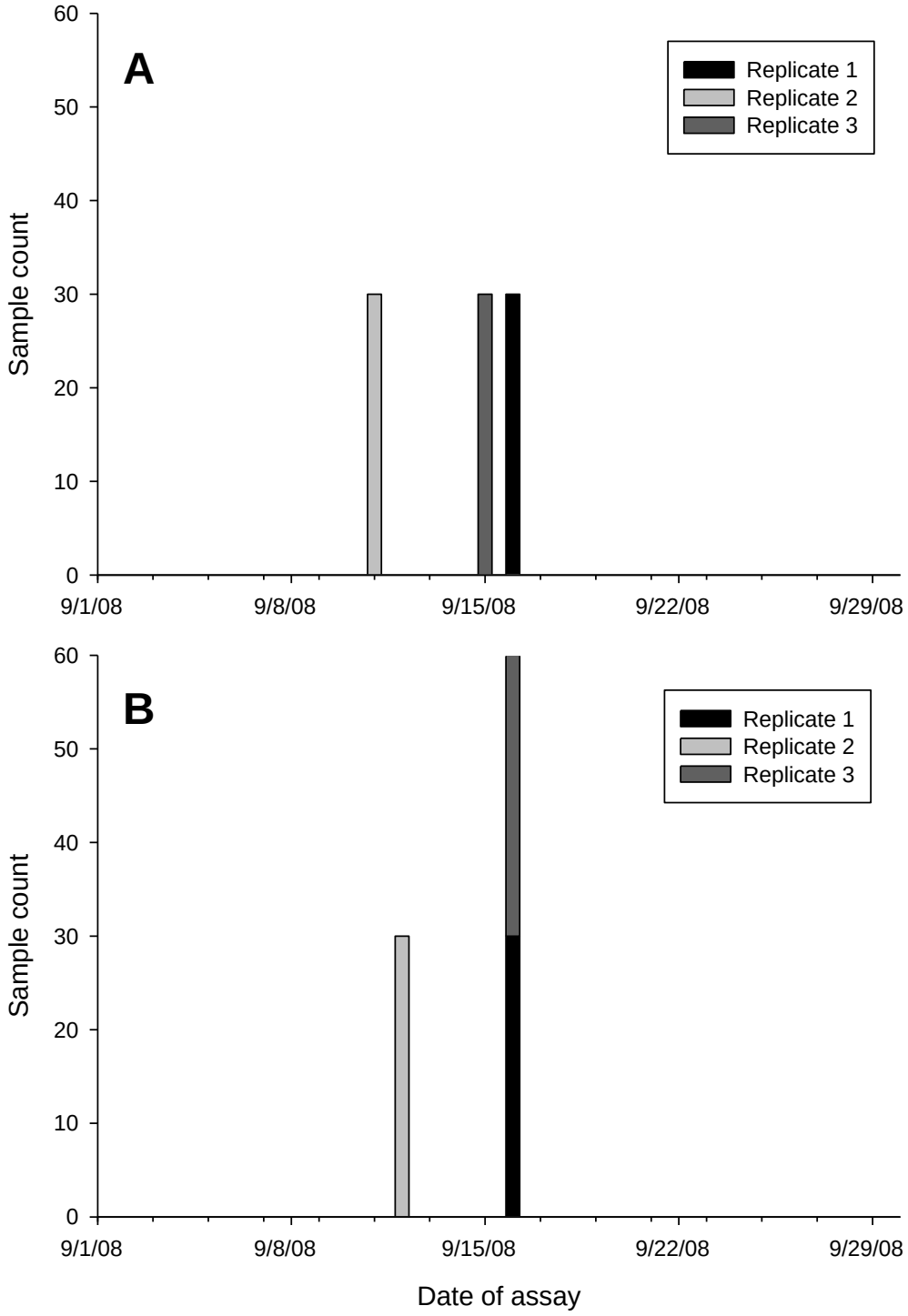


Fig. A7. Distribution of quail plasma samples from methamidophos simulated field exposures within cholinesterase assays by experimental replicate and date for both the pre-exposure (**A**) and post-exposure (**B**) sampling periods.

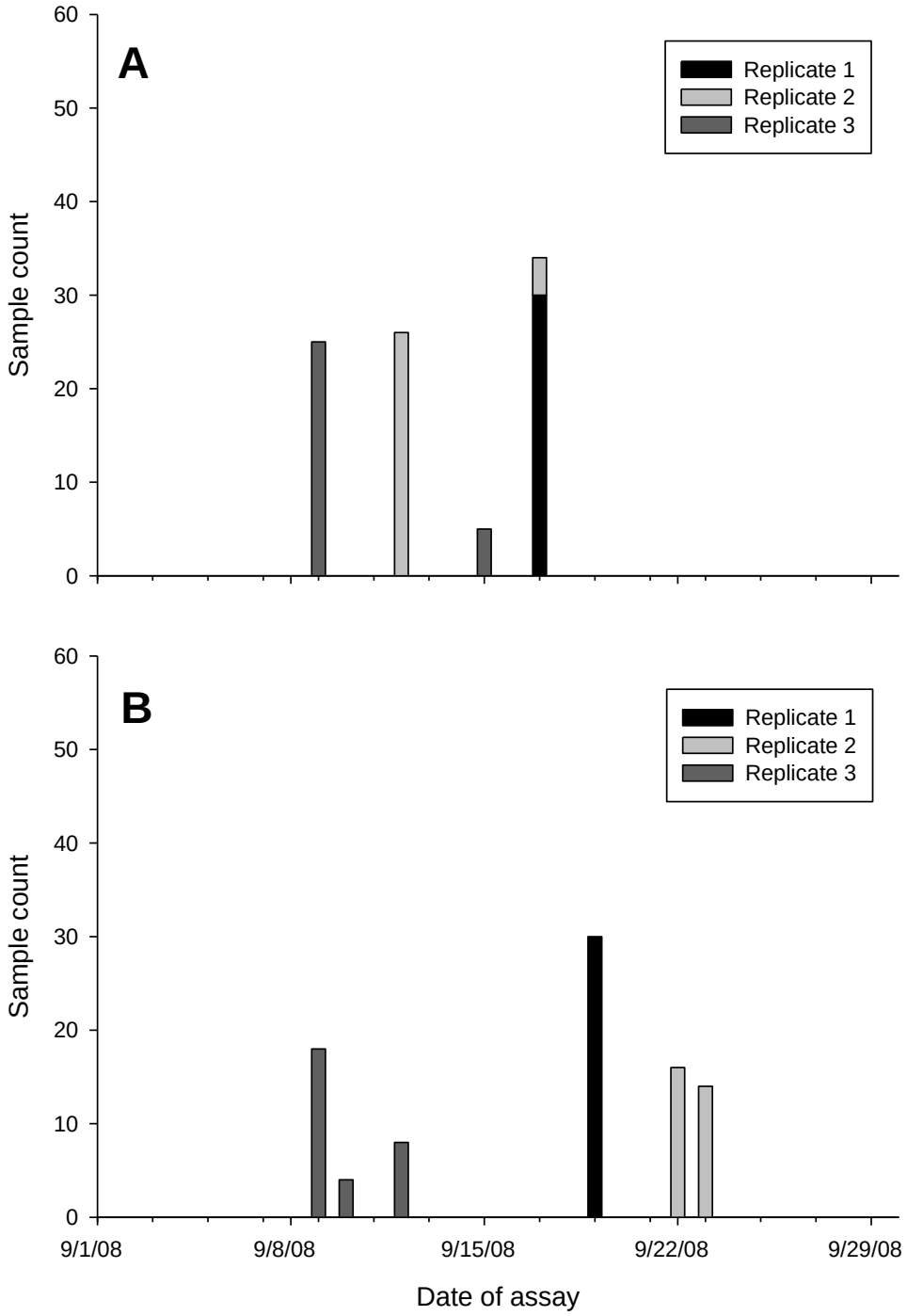


Fig. A8. Distribution of quail plasma samples from naled simulated field exposures within cholinesterase assays by experimental replicate and date for both the pre-exposure (**A**) and post-exposure (**B**) sampling periods.