Toxicological evaluation of inhalation exposure to benzene and toluene in a raptorial bird, the American kestrel, *Falco sparverius*.

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By

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PREFACE

This thesis has been organized as a series of manuscripts that will be submitted for publication in scientific journals. Some repetition of introductory and methodological material was unavoidable.

ABSTRACT

Benzene and toluene are representative volatile organic compounds (VOCs) released during production, storage, and transportation associated with the oil and gas industry. Benzene and toluene are chemicals of concern because they are released in greater and possibly more biologically significant concentrations than other compounds.

Most studies of air pollution in high oil and gas activity areas have neglected to consider risks to top-level predators. Birds can be used as highly sensitive monitors of air quality. Since the avian respiratory tract is physiologically different from a rodent respiratory tract, effects of gases cannot be safely extrapolated from rodent studies. I hypothesized that benzene, being haematotoxic and immunotoxic, along with the neurological and possible endocrine disrupting effects of toluene would be more toxic in birds than in mammals.

After two summers of experimental exposure of wild and captive American kestrels to high (10ppm and 80ppm) or environmentally relevant (0.1ppm and 0.8ppm) levels of benzene and toluene, respectively, altered immune, haematopoeitic, behavioural, and endocrine responses characteristic in mammals, were evident in the kestrels.

There was a decreased cell mediated immune response as measured by delayed type hypersensitivity tests in all exposed birds ($p = 0.028, 0.004$). An increase in humoral immunity as compared to control individuals ($p = 0.041, 0.031$) was also apparent in both dose groups. Plasma retinol levels were decreased in 2005 and 2006 high dose individuals ($p = 0.008, 0.048$).

The majority of haematopoeitic effects involved the erythroid lineage in the bone marrow and the polychromatophilic erythrocytes systemically. There were no significantly adverse responses in the bone marrow with regards to the granuloid lineage but systemically there was a prominent eosinophilia ($p = 0.045$) and basophilia ($p = 0.006$) in low exposure groups. The loss of communication between polychromatophilic erythrocytes in the post-mitotic pool within the bone marrow and the peripheral blood was present in low and high exposure individuals compared to control birds ($p = 0.013, 0.402, 0.974$). The number of polychromatophils in the circulation of low dose group individuals was decreased compared to control birds ($p = 0.029$). This may be a function of toluene's inability to inhibit biotransformation enzymes at low concentrations leading to blood cell targeting by benzene's increased phenolic metabolite production. This theory is corroborated by the possible decreased benzene metabolism and increased toluene distribution manifesting as increased aggressive responses such as wing beating and vocalization time in the high dose group ($p = 0.025, 0.086$).

The work here has shown American kestrels are sensitive to the air contaminants, benzene and toluene. The present study illustrates the need for reference concentrations for airborne pollutants that are calculated based on data measuring sensitive endpoints specific for avian models. Future studies should evaluate immune, haematopoeitic, and behavioural endpoints, as well as develop more sensitive isoform specific enzyme activity assays to further determine the susceptibility of birds to inhaled toxicants.

ACKNOWLEDGEMENTS

Where to start? First I need to thank and acknowledge the woman who made this all possible, Judit Smits. You have built the foundation that I will build scientific career on. Not only are you an amazing mentor you are a valued friend. Thanks for always listening to my ideas. Brenda Trask a beautiful woman in every sense of the word. When I thought I couldn't do one more ELISA you were there to laugh with me and encourage me. Thank you for everything you taught me in the lab and out. Martin Stoffel, the bird man. Thanks for making me the bird lady. I will never drive the road to Prince Albert and not think of you. Lastly, my beautiful, magnificent, rowdy birds. Thanks for all you gave.

I would also like to thank Nexen, CNRL, and The Wildlife Health Fund (WCVM) for funding as well as the Toxicology Graduate Program for my stipend.

DEDICATION

This thesis is dedicated to many people, who, without their love and support this work would never have been completed and I would be sitting on a beach in Thailand.

Kristopher, an amazing person to share my life with and the greatest sciencologist I know.

Mom and Dad, what can I say, you've been there through it all and heard everything…..except…. I finally finished.

Grandma Lorna, my pillar of strength.

“The wildlife of today is not ours to dispose of as we please. We have it in trust, and must account for it to those who come after”
King George VI of England

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LIST OF ABBREVIATIONS

BROD	Benzyloxyresorufin-o-dealkylase
BSA	Bovine Serum Albumin
CMI	Cell-Mediated Immunity
CYP450	Cytochrome P450 Monoxygenase System
DNP-KLH	Dinitrophenol-Keyhole Limpet Hemocyanin
DTH	Delayed Type Hypersensitivity
ELISA	Enzyme Linked ImmunoSorbent Assay
EROD	Ethoxyresorufin-o-dealkylase
GHO	Great Horned Owl
GLM	General Linear Model
GMI	Granuloid Maturation Index
HMI	Humoral Mediated Immunity
HPLC	High Performance Liquid Chromatography
LOAELs	Lowest Observable Adverse Effect Levels
LSD	Lowest Significant Difference
MROD	Methoxyresorufin-o-dealkylase
NADPH	Nicotinamine Adenine Dinucleotide Phosphate
OCs	Organochlorine Pesticides
OD	Optical Density
PAHs	Polycyclic Armoatic Hydrocarbons
PBDEs	Polybrominated Diphenyl Ethers
PBS	Phosphate Buffered Saline
PCBs	Polychlorinated Biphenols
PCV	Packed Cell Volume
PROD	Pentoxyresorufin-o-dealkylase
PTFE	Polytetrafluoroethylene
PVC	Polyvinyl Chloride
RA	Retinoic Acid
RBCs	Red Blood Cells
RH	Relative Humidity
ROS	Reactive Oxygen Species
RP	Retinyl Palmitate
SDS	Sodium dodecyl sulfate
T3	Triiodothyronine
T4	Thyroxine
THs	Thyroid Hormones
TRs	Thyroid Receptors
TTR	Transthyretin
VOCs	Volatile Organic Compounds
WBC	White Blood Cell

CHAPTER 1

GENERAL INTRODUCTION

1.1 Oil and Gas Industry Background

1.1.1. Activity in Saskatchewan

The Western Canadian Sedimentary Basin (WCSB) covers a 1.4 million km^2 (550,000 $miles^2$) area that encompasses southwestern Manitoba, southern Saskatchewan and Alberta (Porter *et al.* 1982). It is essentially a massive wedge of sedimentary rock 6 km thick that contains one of the world's largest reserves of petroleum and natural gas and is a major supplier of the North American market energy requirements. Through the exploitation of the WCSB Saskatchewan has become Canada's second largest oil producer at 152.9 million barrels in 2005 with an approximated 1.9 billion dollars invested and 1.1 billion dollars generated in revenue (Saskatchewan Industry and Resources 2006). Historically Saskatchewan has produced 4.4 billion barrels since the discovery of oil in 1944 and there are an approximated 1.2 billion barrels of in ground oil remaining (Saskatchewan Industry and Resources 2006). In light of the potential for economic growth, the upstream oil and gas industry has intensified operations in Saskatchewan. The result is a changing Saskatchewan and Alberta landscape that is dotted with wells, batteries, and flares, while being cross tracked by pipelines.

1.1.2 Airborne Oil and Gas Production Emissions

The methods by which oil and gas are obtained, stored, refined, and transported can all lead to atmospheric releases of potentially toxic airborne contaminants, such as, benzene and toluene. There are two main contributors to the release of air pollutants from these processes. The first, fugitive emission is classified as the leaking of raw, unprocessed gas from connections or valves. Occurrences are primarily from well or compressor sites, gas processing plants, and drilling procedures. The second source of volatile contaminants is from venting and flaring which involves the intentional release of gas or byproducts of incineration in an attempt to prevent potential explosions and produce less or nontoxic by-products (PCF 2000; Leahey *et al.* 2001; Shewchuck 2002).

Two recent studies determined that open flares operate at 68% (Leahey *et al.* 2001) and 84% (PCF 2000) efficiency. Inefficient or incomplete combustion of gas due to air turbulence, temperature fluctuations, or improper air mixing results in a black or grey plume composed of; carbon monoxide, unburned hydrocarbons, volatile organic compounds (benzene, toluene, ethyl benzene, xylene), polycyclic aromatic hydrocarbons (PAHs), H_2S, carbon disulfide, carbonyl sulfide, oxides of nitrogen, polycyclic organic matter, and particulate matter like ash and soot (Strosher 2000; PCF 2000; Leahey *et al.* 2001). Many of these compounds are associated with adverse health effects. For example benzene is carcinogenic and CS_2 is a central nervous system toxicant (Verma *et al.* 2000).

1.2 Volatile Organic Compounds (VOCs)

Chemically the VOCs are unsaturated cyclic hydrocarbons (aromatic hydrocarbons) that contain one or more benzene rings. These chemicals have high enough vapour pressures to enter the atmosphere under normal temperatures and

atmospheric conditions (ATSDR 2005). Benzene, toluene, ethylbenzene, and xylene (BTEX) are often used as representatives of this group. They are primarily derived from petroleum distillation and the inefficient or incomplete combustion of waste natural gas or solution gas (Verma *et al.* 2000). These solvents are immune, blood, and nervous system toxicants, as well as proven or suspected agents of carcinogenicity (ATSDR 2000; ATSDR 2006). Considering the likelihood of exposure, potential sources, and associated toxicities, the VOCs have been designated chemicals of concern.

1.2.1 Benzene

1.2.1.1 Pharmacokinetics

Inhalation is the primary route of exposure to benzene (U.S.Public Health Service 2005; ATSDR 2006). Following inhalation, benzene is distributed throughout the body preferentially moving into adipose tissue and bone marrow, the primary target tissues, due to its lipophilicity (Irons *et al.* 1979a).

Benzene elimination appears to follow a two compartment model that varies with species. The initial half-life in rats is approximately 42 minutes and the secondary, 13.1 hours (Rickert *et al.* 1979).These data can not be accurately extrapolated to birds as the lung physiology and enzymatic capabilities differ greatly from those in mammals. The half-life of benzene varies depending on the benzene concentration, duration of exposure, and species exposed.

Benzene can be metabolized by various enzymatic systems in the lung, liver, or bone marrow to reactive metabolites that cause both cancerous and non-cancerous effects (Rana and Verma 2005). The first step in benzene metabolism is the formation of benzene oxide, an epoxide by the biotransformation enzyme cytochrome P450 (CYP450)

isoform 2E1. Many tissues have this metabolic capability but the liver and lung are responsible for the majority of metabolites formed (Powley and Carlson 2000).There are at least two metabolic pathways proceeding from this intermediate (Snyder and Hedli 1996; Snyder 2004).

A non-enzymatic rearrangement may occur to form phenol. Phenol has numerous biotransformation pathways, the most important being CYP2E1. This pathway will produce hydroquinone or catechol, which are subsequently oxidized via myeloperoxidases to one of two forms of benzoquinone or catalyzed by CYP2E1 again to form benzenetriol. These reactive intermediates will distribute to the bone marrow (Irons *et al.* 1979b; Abraham 1996). Each of the phenolic metabolites (phenol, catechol, and hydroquinone) can also undergo sulfonic or glucuronic conjugation and be excreted in the urine. The primary excretion products are S-phenylmercapturic acid, and *trans, trans*-muconic acid (Abraham 1996; Weisel *et al.* 1996; Snyder 2004).

If the CYP2E1 pathway is not entered, benzene oxide is converted by glutathione to S-phenylmercapturic acid or alcohol dehydrogenase and aldehyde dehydrogenase to *Trans, trans*-muconic acid (ATSDR 2006). Metabolites or the parent form are excreted in the urine or released through the lungs.

The main biotransformation enzyme when high concentrations of benzene are present is CYP2E1. In comparison CYP2B1 is more metabolically important at low concentrations (Gut *et al.* 1993; Gut *et al.* 1996b). CYP2F2 also plays an important role but mainly in pulmonary tissues. CYP2E1 has a higher maximal rate of metabolism but a lower affinity for benzene than CYP2F2 (Gut *et al.* 1996a).

The key to understanding benzene's toxicity is the phenolic products of cytochrome CYP450 metabolism, which are more toxic than the parent form (Rana and Verma 2005; Morgan and Alvares 2005). Once phenol is bioactivated to benzoquinone and hydroquinone these can enter a reduction-oxidation (re-dox) cycle in the bone marrow producing destructive reactive oxygen species (Snyder and Hedli 1996; Gut *et al.* 1996a; Snyder 2004).

There are two important concepts to consider when designing any study that involves benzene. Firstly pulmonary biotransformation enzymes within microsomes convert benzene to hydroquinone more effectively than the hepatic counterpart which may add to toxic metabolite load (Chaney and Carlson 1995; Powley and Carlson 1999; Powley and Carlson 2001; Sheets and Carlson 2004). Secondly, intermittent exposure to low benzene concentrations has proven more toxic than single high exposures based on tumor formation in mice (Cronkite *et al.* 1989).

1.2.1.2 Toxicity in Mammals

Mechanism of Toxicity

The reactive metabolites (phenol, hydroquinone, catechol and benzenetriol(s)) are transported from the liver or lung to the bone marrow (Tunek *et al.* 1981; Tunek *et al.* 1982; Morgan and Alvares 2005).Once in the bone marrow, myeloperoxidases convert the metabolites to highly reactive semiquinone radicals that stimulate the production of reactive oxygen species (ROS).The ROS may damage cellular macromolecules leading to granuloid and erythroid progenitor cell dysfunction (Tunek *et al.* 1981). The lowest observable adverse effect levels (LOAELs) for immunological effects of benzene have been estimated at 10 ppm (Rozen *et al.* 1984a).

Haematopoietic System

Precursors of all major types of blood cells are susceptible: erythrocytes, lymphocytes, thrombocytes and granulocytes. This can lead to pancytopenia (reduction in all types of blood cells), aplastic anemia from severe depression of bone marrow function, or a neoplastic response with acute myelogenous leukemia (Rana and Verma 2005).

Immune System

Immunologically benzene has been associated with adverse effects on both arms of the acquired immune system, humoral (B lymphocytes) and T lymphocyte mediated, as well as innate immunity (Snyder *et al.* 1993). Benzene concentrations as low as 10 ppm have decreased the formation of B-lymphocytes, suppressed immunoglobulin production, and inhibited the spleen from releasing mature T-lymphocytes (Rozen *et al.* 1984a).

In addition to alterations in the maturation of lymphocytes, toxic metabolites may inhibit lymphocyte proliferation and agglutination by reacting with intracellular sulfhydral groups through ROS (Wells and Nerland 1991).This could alter membrane fluidity and lead to decreased lymphocyte function as well as decreased circulating populations. Studies in mammals have shown that B lymphocytes were more sensitive to benzene related toxicity than T lymphocytes (Stoner *et al.* 1981).

1.2.2 Toluene

1.2.2.1 Pharmacokinetics

Studies on laboratory animals and humans have shown that toluene is readily absorbed from the respiratory tract with an uptake of 40 – 60% (Carlsson 1982). The half

life ranges form 12 to 65 hours (Carlsson 1982). Following absorption, toluene is rapidly distributed between the plasma and red blood cells (RBCs) (Lam *et al.* 1990).The association of toluene with the hemoglobin of RBCs increases the transport of toluene to all areas of the body including the brain, liver, and lung. Toluene has a great affinity for the lipid-rich white matter areas of the brain compared to areas high in grey matter (Ameno *et al.* 1992).

Once absorbed, toluene like benzene is preferentially metabolized by the CYP450 system. CYP2E1 will biotransform toluene to benzyl alcohol and CYP1A2 will form *ortho* and *para* cresols following a toluene epoxide intermediate (Nakajima *et al.* 1997). Benzyl alcohol is then converted by alcohol and aldehyde dehydrogenases to form benzoic acid which may be conjugated with glycine to form hippuric acid or catalyzed by UDP-glucuronyl transferase to form benzoyl glucuronide (Nakajima and Wang 1994).

The toluene epoxide intermediate, which can form *o* or *p* cresol, will eventually be conjugated by sulfates and glucuronides. The other pathways may produce S benzyl mercapturic acid. All three of these products will be excreted in the urine (Duydu *et al.* 1999; Pierce *et al.* 2002).

The most common urinary metabolites are hippuric acid, benzoyl glucuronide, *ortho* and *para* cresol, and S-benzyl mercapturic acid (Doorn *et al.* 1980; Lof *et al.* 1993; Duydu *et al.* 1999). Parent toluene may be excreted in exhaled air. Approximately 20-25% of inhaled toluene is absorbed and retained in the parent form (Lof *et al.* 1993). This retention of parent toluene is important as it is the more toxic form which will be distributed to lipid rich areas.

1.2.2.2 Toxicity in Mammals

Mechanism of Toxicity

The neurological effects of toluene exposures have been attributed to the reversible interactions between toluene and lipid or protein components of nervous system membranes (Greenberg 1997).On a more molecular level the interaction is thought to be the intercalation of toluene into the lipid bilayer leading to disruption of the membrane function (Franks and Lieb 1985). Mechanistically it appears that the repeated interaction of toluene with membrane proteins can change activities involved in the synthesis or degradation of glucocorticoids and neurotransmitters leading to synaptic dysfunction (Fuxe *et al.* 1982;Voneuler *et al.* 1993). The LOAEL for neurological effects associated with toluene exposure has been estimated at 80 ppm (Voneuler *et al.* 1993; Hilleforsberglund *et al.* 1995).

Respiratory System

As well as increased lung weights, irritation of the upper airways and degeneration of the nasal epithelium have been associated with animal exposures to toluene (Poon *et al.* 1994). Erosion and metaplasia of the olfactory epithelium and degeneration of respiratory epithelium were seen in rats but not mice (ATSDR 2000). The effects of toluene are species dependent and extrapolation to other test species is difficult.

Haematopoietic system

Toluene does not appear to affect the hematological system. Early studies indicated an effect but this has since been attributed to benzene contamination of exposure samples (Ukai *et al.* 1993; Poon *et al.* 1994).

Endocrine

Hypothyroidism has been diagnosed after chronic exposures in humans (Hong *et al.* 1996). Animal studies have been far less conclusive. (Poon *et al.* 1994) witnessed thyroid alterations but other studies have not reproduced these findings.

Nervous System

Dysfunction of the central (CNS) nervous system is a critical health concern following acute, intermediate, or chronic toluene exposure. Neurotoxic effects in laboratory animals include excitatory and depressant CNS symptoms in humans (Bruckner and Peterson 1981a;Bruckner and Peterson 1981b), decreases in response time and accuracy in monkeys (Taylor and Evans 1985), nystagmus (involuntary eye movement) (Larsby *et al.* 1986) and decreased shock avoidance behaviour in rats (Kishi *et al.* 1988), and moderate levels of exposure in rats have resulted in loss of outer hair cells leading to loss of hearing in the high frequency range (Pryor and Rebert 1992). In animals, these behavioural changes have been associated with changes in dopamine and norepinephrine brain neurotransmitter levels (Voneuler *et al.* 1989). Changes in glial fibrillary acidic protein, a structural marker for astrocytes have also been noted (Little *et al.* 1998a).

1.2.3 Benzene and Toluene Interaction

The metabolic interaction of benzene and toluene *in vitro* and *in vivo* in rats has been described as a dose dependent competitive inhibition. The absorption and distribution are unaffected by the presence of one another (Sato and Nakajima 1979).

The co-administration of benzene and toluene decreases adverse bone marrow effects including chromosomal aberrations and depression of erythrocyte, leukocyte, and

thrombocyte production. The evidence supports the theory that co-exposure results in decreased formation of hydroxylated benzene metabolites due to inhibition of the phenol producing pathway (Medinsky *et al.* 1994a). It has also been reported that hippuric acid and *o*-cresol excretion is decreased when benzene is present. This implies that benzene is also inhibiting the metabolism of toluene (Brondeau *et al.* 1992).

A binary exposure has very different physiological implications than does a single chemical exposure. As the quantity of phenolic metabolites produced from benzenes biotransformation decreases the bone marrow damage is less pronounced (Snyder 2004). In contrast toluene is toxic in the parent form. As hippuric acid and o-cresol decrease in the urine the parent compound is being retained in the body and neurological effects will be expected to increased (Purcell *et al.* 1990b). To further complicate the situation toluene more effectively interferes with benzene metabolism than the reverse (Andrews *et al.* 1977).

The effects are therefore dose dependent on two levels. The respective concentrations of benzene and toluene will differentially affect each others metabolism (Little *et al.* 1998b) just as the presence or absence of either chemical does (Purcell *et al.* 1990a).

1.3 Avian Risk Factors

1.3.1 Air Quality Issues

Concerns relating to human contaminant exposures in association with oil and gas emissions are well documented and numerous epidemiological studies have been conducted (Schechter *et al.* 1989; Schechter *et al.* 1990; Kilburn and Warshaw 1995). Risks to wild birds, however, have historically been studied on a much smaller scale. The

American kestrel and other larger falcons are abundant in the prairie regions and are thus exposed in a similar fashion as any human inhabiting these areas (Hoffman and Smith 2003).

Upon observation of several open flares in Southern Saskatchewan it was noted that certain species of falcons were nesting in close proximity. Many falcons are repeat breeders with high fidelity to breeding territory (Janes 1984) and decreases in certain species of prairie falcons have been documented (Kirk and Hyslop 1998; Hoffman and Smith 2003). These two concepts coupled with the unique respiratory physiology of birds, which results in increased absorption of gases from inspired air relative to mammals (Brown *et al.* 1997), make it plausible that the components of flare gas are having greater toxicological effects on certain raptor populations as compared to humans (Schechter *et al.* 1989; Schechter *et al.* 1990), beef cattle (Waldner *et al.* 2001; Waldner 2001), and European starlings (Carmalt 2005).

1.3.2 Exposure Potential

The toxicological effects associated with benzene and toluene exposures in mammals have been shown to increase with intermittent low dose exposures (Cronkite *et al.* 1989). This pattern of exposure is similar to the migration and reproductive cycle of many species of falcons that use the Central or Prairie flyway, a major North American migration route (Schmutz *et al.* 1991) to reach their Saskatchewan and Southern Alberta (prairie and boreal plains ecozones) breeding grounds (Amiro *et al.* 2001). This also overlies the geological formation known as the WCSB, a highly exploited region with regards to the oil and gas industry. Figures 1-1 and 1-2 depict the area covered by the aforementioned regions. Raptors known to nest in Saskatchewan and Alberta include but

are not limited to; American kestrel (*Falco sparverius*), Prairie Falcon (*Falco mexicanus*), Peregrine falcon (*Falco peregrinus*), Merlin (*Falco columbarius*), Swainson's hawk (*Buteo swainsonii*), Red tailed Hawk (*Buteo jamaicensis*), Ferruginous Hawk (*Buteo regalis*), Northern Harrier (*Circus cyaneus*), and Osprey (*Pandion haliaetus*) (Schmutz *et al.* 1991). Individuals nesting in prairie and boreal plain ecozones could potentially be exposed to high concentrations of air contaminants at critical life stages such as early development in young and periods of increased stress and high biological demands in adults.

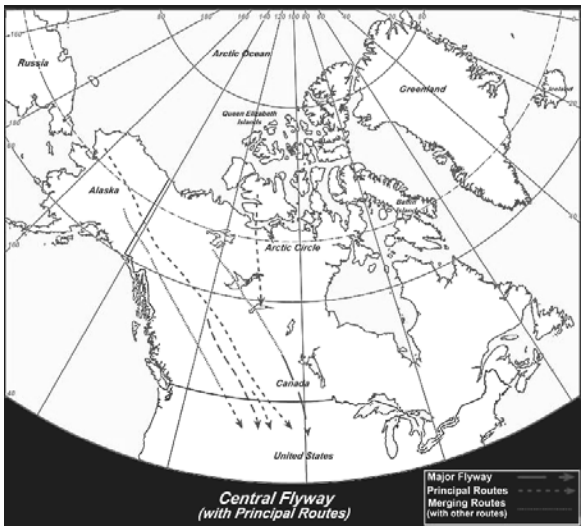

Figure 1-1 Central Flyways with principal routes used by North American raptors, www.birdnature.com, 2006.

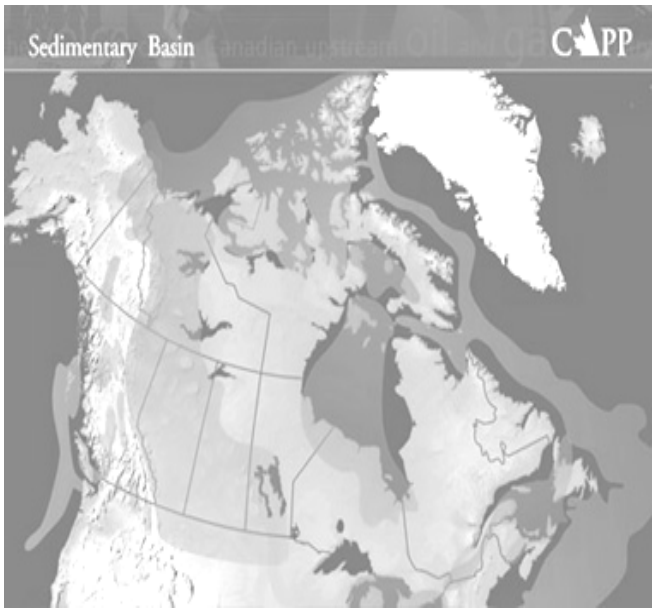

Figure 1-2 Range of the Western Canada Sedimentary Basin in Alberta, Saskatchewan, and Manitoba© (Canadian Association of Petroleum Producers, CAPP, Calgary, AB Canada).

1.3.3 Population Trends

Observations from 1959 -1988 (Kirk and Hyslop 1998) and 1977-2001 (Hoffman and Smith 2003) examined population trends of Canadian and Western North American raptors in many regions including the prairies and boreal plains. These studies documented significantly negative population trends for 7 species of raptors (2 owl, 5 falcon) in these regions over the past 25 years (Hoffman and Smith 2003). The Northern harrier is the only species to have decreased in both eco-zones. Within the Prairie eco-zone the Bald eagle, Swainson's hawk, and American kestrel have all shown decreased population trends. The Merlin, Great horned owl, and Short-eared owl have decreased significantly in the boreal plains (Kirk and Hyslop 1998).

These moderate to long-term data are useful in assessing the magnitude of highs and lows of population changes and true population declines. Declines can be due to a

variety of factors such as temperature, habitat loss, human population, and natural highs and lows. It is also important to consider pollution and unfavourable breeding conditions as possible factors. Kirk and Hyslop (1998) arrive at the conclusion that declining populations in the prairies need to be investigated due to the heavy use of pesticides. I have taken this one step further and considered the possibility that poor air quality due to VOCs and toxic components of flare gas within the WCSB and prairie flyway area could be contributing to the declines.

1.4 Avian Physiology

1.4.1 Respiratory System

There are many distinct morphologic, physiologic, and mechanical differences between the avian respiratory system and the mammalian lung. Historically humans have used birds as an early warning system and valuable experimental models for monitoring air quality (the canary and the coal miner) (Loranger *et al.* 1994; Brown *et al.* 1997). It can be inferred from this that the anatomical and physiological differences of the avian respiratory tract make it more sensitive and responsive to inhaled substances than the mammalian lung.

The seven to nine air sacs of the avian respiratory system act as a pump moving air through the bird's nearly constant-volume lung. The air sacs fill simultaneously during inspiration and empty during expiration making them an air flow tool not used for gas exchange (Scheid *et al.* 1972; Brown *et al.* 1997). The system maintains a unidirectional flow through the major gas exchange area of the bird's lung. During inspiration nearly all the gas passes caudally through the intrapulmonary bronchus, half passing to the caudal air sacs and half entering the lung via the dorsobronchi. During expiration that same

breath exits the caudal thoracic and abdominal air sacs moving cranially. Differences in the surface density of gas exchange tissue, the thickness of tissues, and the presence of surfactant have enabled the bird to separate gas exchange from the site of tidal expansion.

Mammalian bronchi ramify like the roots of a tree throughout the pulmonary parenchyma and terminate in single blind ended alveoli where gas exchange and tidal expansion occur (Brown *et al.* 1997). The birds primary bronchi in contrast has 2 clusters of secondary laterobronchi, 4 ventrobronchi cranially, and 7-14 dorsobronchi caudally which divide into parallel tertiary bronchi called parabronchi where gas exchange will occur (Maina 2000a). The parabronchi complete an airway loop and are arranged in a hexagonal array that resemble a honeycomb (Scheid and Piiper 1970; Duncker 1972). The lumen of each parabronchus is surrounded by a mantle of gas-exchange tissue consisting of extensive networks of narrow air and blood capillaries. The birds parabronchi is 10 μm thick as compared to the > 300 μm thickness of a mammalian alveoli (Brown *et al.* 1997) . The avian gas-exchange barrier is formed by the air capillaries' squamous epithelium, the blood capillaries' endothelium, and the basal lamina (Brown *et al.* 1997). This is qualitatively similar to that of the mammalian alveolus and its pulmonary blood capillaries (Tenney and Remmers 1963; Maina 2000a). Also in contrast to alveoli, parabronchi lack resident macrophages and are lined with a nonsecretory, nonstratified, nonciliated epithelium (Tenney and Remmers 1963; Brown *et al.* 1997). This implies differences in enzymatic capabilities between avian and mammalian lungs, which may lead to differential responses to inhaled toxicants (Buckpitt *et al.* 1982a).

The sum of these differences and similarities equates to; birds can fly (Maina 2000b), given identical ventilation a bird can take up approximately twice the amount of gas (Brown *et al.* 1997), and birds will differentially metabolize the chemicals present in inspired air as compared to mammals (Buckpitt *et al.* 1982b).

1.4.2 Immune System

The immune system provides surveillance against pathogens, parasites, foreign particles, and cancer cells through a network of soluble circulating proteins, cells, and antibodies (Fairbrother *et al.* 2004). The immune system of all vertebrates depends on specialized microenvironments where pluripotent lymphocytes can differentiate into clones of lymphocytes with the ability to respond to self or foreign antigens. This environment also allows primary lymphocytes to respond to specific antigens and clonally expand (Glick 2000).

Specific immunity which is dependent upon T lymphocyte mediated immunity (CMI) and antibody or humoral mediated immunity (HMI) and the macrophages and natural killer cells of nonspecific immunity interact to produce a fully functioning, protective, and highly conserved immune system (Sharma 1991; Fairbrother *et al.* 2004). The CMI acts through T lymphocytes and effector cells, which regulate the HMI, and nonspecific responses. These T lymphocytes communicate with each other and other cells via cytokines such as interleukins. The HMI is characterized by the capacity to produce antibodies induced by exposure to foreign antigens, such as, vaccines, viruses, bacteria, and toxins. The B lymphocyte of the HMI originates in the Bursa of Fabricius in birds and the T lymphocytes of the CMI originate from the thymus as in other vertebrates (Fairbrother *et al.* 2004).

The bursa is a dorsal diverticulum of the cloaca that will regress before sexual maturity. B lymphocytes produced in the embryonic spleen migrate to the bursa where gene conversion occurs to determine immunoglobulin expression. There are 3 avian forms of immunoglobulins (Ig): IgM (primary antibodies), IgG (secondary antibodies), and IgA (mucosal antibodies) (Glick 2000). When a foreign protein is encountered mature B lymphocytes will migrate out of the bursa differentiate into plasma cells and secrete antibodies that are specific and capable of neutralizing those specific antigens (Sharma 1991).

T lymphocytes originate in are processed by the thymus. The central medulla enclosed by a cortex contains three types of thymocytes (Tizard 1992). The first moves to the spleen and intestine and the other two types will differentiate based on their interactions with self antigens and major histocompatability complex to become T helper cells (CD4) or T suppressor lymphocytes (CD8). T lymphocytes produce cytokines that act as chemical messengers that may enhance B lymphocyte, other T lymphocyte, macrophage or granulocytic (neutrophil, eosinophil, basophil) responses (Fairbrother *et al.* 2004).

Immunomodulation is any change in the body that results in an activation or suppression of the immune system. Environmental pollutants have been associated with many immunomodulatory events in a wide range of wildlife species. PCBs have been associated with antibody and cell mediated immune modulation in avian species (Smits and Bortolotti 2001; Smits *et al.* 2002), and the ingestion of petrochemicals from oils spills has resulted in hypercellularity of erythroid progenitors in the bone marrow and reduced circulating leukocytes (Briggs *et al.* 1997).

1.4.3 Endocrine System

Endocrine disruption by environmental pollutants can be achieved in one of three ways. Chemicals can bind receptors and act as mimics to endogenous ligands, they can alter the synthesis or metabolism (through cytochrome P450 enzyme activity alteration or binding transthyretinthe carrier protein for thyroxine and retinol), and lastly they can interfere with the hypothalamus-pituitary-endocrine gland axis signaling (Dawson 2000).

1.4.3.1 Thyroid hormones

In homeotherms such as birds, thyroid hormones (THs) have multiple effects on metabolism and development. Thyroid hormones regulate the basal metabolic rate, maintain constant and high temperatures, stimulate growth and differentiation, and affect protein and lipid metabolism by acting catabolically at high concentrations and anabolically at low concentrations (Decuypere *et al.* 2005).

Triidothyronine (T3) is formed by the irreversible mono-deiodination of the inner or outer ring of the primary secretory product of the thyroid gland, thyroxine (T4). Thyroxine 5'-deiodinase types I and II are the enzymes responsible for the conversion of T4 to T3. Thyroxine is relatively inactive in comparison to T3 due to its low binding affinity for thyroid receptors (TRs) and serves as a regulator of metabolism due to its availability for transformation to T3. If deiodination of the outer ring occurs the pathway is activated, the inner ring subsequently leads to inactivation (Brent 1994). In order for THs to enter a cell or organelle they must bind high affinity transporters such as organic anion transporter proteins on plasma membranes (Decuypere *et al.* 2005). Once in the nucleus THs can bind one of two nuclear TRs and subsequently modify gene expression. The DNA sequences that bind the T3 receptors will determine which genes are stimulated

or inhibited. These receptors are expressed at different developmental stages and can increase or decrease the amount of T3 bound and subsequent gene products (Brent 1994).

The concentrations of T3, T4, deiodinases, and the number and type of available TRs will affect the systemic status of THs. If an adverse event occurs due to a chemical, physiological stressor, or genetic anomaly hyperthyroidism or hypothyroidism may present. This may adversely affect plumage production (Quinn *et al.* 2005), cardiovascular function, hepatic and adipocyte lipid metabolism and gene expression, pituitary hormone secretion, muscle contraction, and neural development (Rolland 2000; Colborn 2002; Mcnabb 2003).

Lab and field studies of environmental pollutants have examined the effects of suspected thyroid hormone disruptors. Polychlorinated biphenyls (PCBs) in the American kestrel (Smits *et al.* 2002; Quinn *et al.* 2002; Quinn *et al.* 2005), Ammonium perchlorate in Bobwhite quail chicks (Mcnabb 2003; Mcnabb *et al.* 2004), and Polybrominated diphenyl ethers (PBDEs) in American kestrels (Fernie *et al.* 2005b) have all identified a degree of thyroid hormone suppression in relation to environmental contamination. Toxicity can also be related to histopathological changes and gross lesions of thyroid glands as shown by organochlorine residues (OCs) (Fox *et al.* 1998) and PAHs (Moccia *et al.* 1986a) in Herring gulls.

1.4.3.2 Vitamin A

Vitamin A is defined as any compound possessing the biological activity of retinol (all-trans retinol, retinyl palmitate (RP), 11-cis and 13-cis retinoic acid, B carotene) that is essential for life in chordates (Blomhoff and Blomhoff 2006). No animal species can synthesize vitamin A so it must be acquired from the diet.

The most important functional isoform of Vitamin A is retinoic acid (RA), where as the liver storage form is retinal palmitate (RP). All-trans retinol once sequestered from the liver and biotransformed from RP can be converted intracellularly to RA which can then bind to retinoic acid receptors and act as a hormone (Ross 2004). The receptor interacts with specific nucleotide sequences of DNA directly affecting gene expression and transcription (Blomhoff *et al.* 1992b). It is RA that is responsible for epithelial cell differentiation and cell surface maintenance, immune competence, bone growth, spermatogenesis, embryonic development, and growth (West *et al.* 1991; Blomhoff *et al.* 1992a; Krinsky 1994; Friedman and Sklan 1997; Evans 2005).

Endocrine disrupting chemicals altering vitamin A status are well documented. Colonies of Herring gulls on the Great lakes had significantly lower plasma retinol and liver RP levels after exposure to 2,3,7,8-TCDD (a common dioxin) (Spear *et al.* 1992), PCBs, DDE, dieldrin, and mirex (Fox *et al.* 1998). Populations of Great Blue Herons in the St Lawrence River area (Boily *et al.* 1994) and Common terns in Belgium and the Netherlands (Murk *et al.* 1996) exhibited decreased egg yolk RP levels after exposure to PCBs and PAHs. PAHs have also been linked to decreased plasma retinol levels in Tree swallows (Bishop *et al.* 1999) and decreased RP levels in adult Herring gulls (Moccia *et al.* 1986b) on the Great lakes. Any deviations in the formation of RA, availability of RP stores, RBP levels, and other conversion factors can lead to dysfunction in any of the previously mentioned biological systems and processes.

1.4.4 Haematopoietic System

This section will deal only with red blood cell (erythropoiesis) and granulocyte (granulopoiesis) production in the bone marrow of the domestic chicken. The maturation and development of lymphocytes was described in section 1.4.2.

The bone marrow is the site of erythrocyte and granulocyte differentiation and B lymphocyte maturation in birds and is often used as an indicator of blood problems associated with disease and toxicant exposure (Dieterlen-Lievre 1988). As an adaptation for flight the majority of avian bones are pneumatic and only certain bones such as the tibiotarsus and sternum contain marrow (Campbell 1995b). Differentiation processes of the three cell lines are compartmentalized within the lumens of sinusoids. One large artery enters through the nutritive foramen and branches to run along side the central vein. The arteries continue to branch into arterioles and finally capillaries that communicate with the sinuses. The sinuses empty directly into the large central vein releasing cells to the systemic circulation (Dieterlen-Lievre 1988). The spleen does not have a haematopoietic function in adult birds (John 1994).

1.4.4.1 Erythropoiesis

A gradient of maturity exists within the sinuses. The more immature erythroblasts and haemocytoblasts (pluripotent precursor cells) are along the periphery (sinus wall) associated with endothelial cells. The more mature polychromatophilic erythroblasts and erythrocytes are positioned in the lumen (Dieterlen-Lievre 1988). The erythroid series matures as follows; rubriblast (erythroblast), prorubricyte, basophilic rubricyte, polychromatic rubricyte, polychromatic erythrocyte, and mature erythrocyte. Immature erythroid precursors are common in the peripheral blood of birds, especially the

polychromatophilic erythroblast (polychromatophil). This cell is smaller than haematocytoblasts and is distinguished by the blue halo of cytoplasm around the nucleus (Dieterlen-Lievre 1988).

Upon maturation avian RBCs retain their nucleus and are ellipsoid in shape. These two features are not present in mammalian RBCs (Dieterlen-Lievre 1988).

1.4.4.2 Granulopoiesis

The visual gradient of maturation is not as pronounced in the granuloid line (heterophils, eosinophils, and basophils). The pluripotent precursors are located along the periphery but are not closely associated with the endothelial cells. In addition the various stages and types of cells in the granuloid series are intermingled among fat throughout the extravascular space (Dieterlen-Lievre 1988). The stages of development include: myeoblast (granuloblast), progranulocyte (differentiation of the three cell types), myelocytes (secondary granulocytes of the heterophil, eosinophil, and basophile lineages appear), metamyelocytes, and finally the mature granulocyte (Campbell 1995a).

Heterophils stain with eosin and their secondary granules are a very distinctive rod shape in American kestrels while the nucleus in multilobate. They are the most numerous granulocyte found in circulation (Dieterlen-Lievre 1988). Avian heterophils are unique in that they are devoid of peroxidase activity. They are known to participate in anti-inflammatory and phagocytic reactions, as well as behave as a bactericide (Maxwell and Robertson 1998).

Eosinophils have round secondary granules with a high concentration of arginine. These cells are the primary effectors during allergic reactions, anaphylaxis and parasitic infections (Dieterlen-Lievre 1988).

Basophils have very distinctive deep purple staining round granules with a unilobular nucleus. These are the least studied of the avian granulocytes and their function is linked to histamine production. They participate in acute inflammation and allergic reactions (Maxwell and Robertson 1995).

1.4.5 Cytochrome P450 monooxygenase system

The cytochrome P450 monoxygenase (CYP450) system is a family of hemoproteins that function within a membrane bound electron transport chain to catalyze the oxidation of many xenobiotics (Sinclair and Sinclair 1997). As a consequence of phase I biotransformation, xenobiotics are chemically changed from a lipophilic compound favouring absorption to a hydrophilic compound favouring excretion. When a volatile compound such as benzene is biotransformed to a more hydrophilic state its elimination rate is slowed and the longer retention time within the body allows benzene to distribute to target organs and potentially increase the level of toxicity. Phase II processes such as sulfonation, glucuronidation, and/or glutathione conjugation may occur after phase I hydrolysis, oxidation, or reduction processes (Casarett and Doull's 2001).

Biotransformation leads to detoxification/activation and excretion/retention of reactive metabolites depending on the xenobiotic. In addition to possible activation and detoxification functions the gene expression of certain CYP450 enzymes such as CYP1A, 2B, 2E1 may be inhibited or induced. If the expression of an enzyme is inhibited the chemical is no longer being biotransformed to an active or inactive form. In contrast, if the expression of an enzyme is being upregulated, the target chemical is more rapidly biotransformed. If the chemical induces or inhibits enzymes that are responsible for its

own biotransformation, it is an auto-inducer/inhibitor, as is the case with both benzene and toluene.

The vast majority of avian specific forms of CYP450 have been characterized in the domestic fowl (*Gallus domesticus*), a species very different due to domestication, from falconiformes or other families within the class Aves. Avian CYP450 forms have been strongly linked to diet and life history. It has been shown that specialized predatory birds have markedly lower activities (Walker 1998). This may be linked to the lack of substrates due to specialized diets or ecological niches. A decreased number of chemicals encountered will negate the need for a wide variety of biotransformation enzymes. In addition, avian CYP450 isoforms do not always biotranform the same chemicals as a similar mammalian form. For example birds have not been responsive to benzyloxyresorufin induction by phenobarbitone, which is a classical inducer in mammals (Lorr *et al.* 1989).

Responses to chemicals have been conserved for at least three classes of inducers in chickens and mammals ((Sinclair and Sinclair 1997; Walker 1998). Planar aromatic hydrocarbons such as benzo(a)pyrene induce a CYP450 family with enzyme activities similar to those of class 1A. Phenobarbitone like drugs induce two forms of CYP450, 2H1 and 2H2. These are 43% homologous with the mammalian 2B family. Lastly an ethanol inducible form with similar characteristics to mammalian CYP2E has been identified (Sinclair and Sinclair 1997). CYP1A, 2B, and 2E homologs have been identified in avian microsomes using immunohistochemical analysis and monooxygenase induction patterns (Walker 1998).

Hepatic mixed function oxygenase activities have been studied in various avian species with a strong focus on seabirds (Rattner *et al.* 1995; Rattner *et al.* 1996). The most widely used assay measures CYP1A activity by ethoxyresorufin-o-dealkylase (EROD) assays. Tree swallows are a common sentinel species that have been studied on reclaimed oil sands (Smits *et al.* 2000), in highly OC contaminated breeding grounds (Papp *et al.* 2005b), in waters polluted with pulp and paper mill effluent (Wayland *et al.* 1998), 2,3,7,8-TCDD (Custer *et al.* 2005) and for PAH pollution from oil refinery leakage (Custer *et al.* 2001).

1.4.6 Neurology and Behaviour

Contrary to the common slur "bird brain" recent research reveals that avian brains are in fact well developed and like primate brains exhibit functional lateralization with left hemisphere dominance. This infers that the bird brain is associated with learning and vocalization (Kamil and Balda 1985; Kamil 1988). Birds have also mastered complex learning problems such as counting and observation (Gill 1995).

If we know the area a xenobiotic will accumulate and exert toxicity we can anticipate the behaviours and brain regions that will be affected. Toluene for example is known to accumulate in lipid rich areas such as the white matter of the brain. In birds it is the midbrain that is comprised primarily of white matter. This region in birds is dominated by two major divisions the optic lobes and the cerebellum (Gill 1995). The midbrain is responsible for regulating vision, muscular coordination, balance, the secretion of neurohormones and the control of seasonal reproduction (Gill 1995). We can hypothesize that any toxicant such as toluene that causes a degeneration of neurons and possible neurotransmitter imbalance could adversely affect any of these vital functions.

The disturbances could manifest as behavioural variations such as unbalanced flight or stooping, inability to focus, improper molting, and/or off-season reproduction.

To study the effects of a behavioural toxicant a battery of tests must be developed that is specific for the animal model. Nesting, perching, hunting, and roosting behaviours have been well documented and studied in American kestrels. Behavioural studies of environmental pollutants such as PCBs (Fisher *et al.* 2006) and electromagnetic field effects (Fernie *et al.* 2000) add to the database of quantifiable behaviours.

1.5 Study Species: American kestrel (*Falco sparverius*)

1.5.1 Natural History

Like all raptors, the American kestrel is sexually dimorphic. The length of the male varies between 21.5–26.5 cm with a wingspan ranging from 51–56 cm and they weigh an average of 103g (Bird 1988).

Kestrels form pairs in which the bond is strong, tending toward permanence. Monogamy is maintained through successive breeding seasons. Returning migrants commonly re-establish territories held the previous year. A cavity nester, kestrels will use holes in trees, rock cavities and crevices in cliffs, artificial nest boxes, or small spaces in buildings. The tendency towards cavity nesting has allowed researchers to successfully use nest boxes to monitor this species throughout the breeding season (Bird 1988).

The Northward migration of kestrels begins in February from South America and March in Mexico. American kestrels move onto the prairies from mid-March to mid-May. They will pair up, establish a nest site, mate, lay eggs, brood, rear young, and fledge in late June and July. The fall migration ranges from mid-July to August although

they can be seen in the prairies until mid-October. American kestrels will not overwinter in the prairie or boreal shield eco-zones (Bird 1988).

1.5.2 Use as Bioindicator Species

Bioindicators act as early warning signs to the nature, severity, and extent of damage an ecosystem is sustaining due to pollution (McCarty *et al.* 2002). Effective monitoring programs are required to determine if industries are operating in accordance with environmental and ecological sustainability criteria set forth by governing bodies (Read *et al.* 2000). Identifying a useful bioindicator species is therefore of strategic importance and forms an integral part of any toxicological and ecological assessment. Birds are considered useful because they are easy to identify, have long life spans, are ecologically versatile, have well known behaviours, and occupy specific niches within food chains (Read *et al.* 2000). American kestrels have been widely and successfully used to monitor the effects of environmental contaminants and are proven acceptable if not highly sensitive bioindicators of environmental pollution (Wiemeyer and Lincer 1987; Smits *et al.* 2002; Bortolotti *et al.* 2003; Love *et al.* 2003; Fernie *et al.* 2005b; Drouillard *et al.* 2007).

1.5.3 Use in Toxicological Studies

Various species of kestrels have become important bioindicators of environmental quality and are used as a test species for comparative toxicology in captive and wild situations. These birds have been used mainly to test the toxicity of OCs, PCBs, metals, and more recently PBDEs (Stendell *et al.* 1989; Yamamoto *et al.* 1998; Love *et al.* 2003; Fernie *et al.* 2005b). The American kestrel serves an important role as a bioindicator and as a raptorial “white mouse”, mainly due to there; small size, non endangered status, ease

with which they are bred in captivity, and taxonomic relationship to other falconiformes (Wiemeyer and Lincer 1987).

1.6 Biomarkers

In the field of environmental toxicology a biomarker is defined as "a xenobiotically-induced variation in cellular or biochemical components or processes, structures, and functions that is measurable in a biological system or sample" (Shugart *et al.* 1992). They may act as early detection systems for a pollutant in the environment by responding to concentrations of a chemical below those known to cause irreversible effects (VanGestel and VanBrummelen 1996). Biomarkers are placed into three classifications: exposure (measurement of the parent compound or metabolite within a compartment of the organism), effect (measurable biochemical, physiological, or other alteration deemed a potential health impairment or disease), or susceptibility (inherent or acquired limitation to respond to a xenobiotic challenge) (Schulte and Mazzuckelli 1991; Holian 1996).

The uncertainty of extrapolation from species to species and individual to population lessens when data is generated from properly designed laboratory studies rather than environmental samples (Schulte and Mazzuckelli 1991). Once the biomarker has been shown to have validity, as well as being reliable, the data generated can assist in quantitative risk assessment. The data can also be used to determine dose response relationships over time, assess the biologically effective dose, allow for interspecies comparisons, and identify species that are at an enhanced risk (Schulte and Mazzuckelli 1991).

1.7 Inhalation Toxicology

Inhalation toxicology refers to the route of exposure and the study of the adverse effects of inhaled gases, aerosols and particles. These studies require more specialized equipment than do oral, subcutaneous, intraperitoneal, or dermal exposure routes. A specific apparatus or inhalation exposure chamber for the controlled delivery of airborne materials is essential for a study to be efficient, effective, and safe for both researchers and test subjects.

There are five basic types of exposure systems; whole body, head only, nose only, lung only, and partial lung (Adkins 1987). Each has its own distinct advantages and disadvantages. The method used in this study will be a whole body exposure system, the most relevant means of exposure for wild birds.

Advantages to using a whole-body exposure chamber include the ability to dose large numbers of animals, accommodate a wide variety of species, and employ minimal restraint (stress reduction). Furthermore it is relatively non-invasive, and chronic studies are feasible (Dorato 1990). Disadvantages include the necessity of large quantities of test material, exposure is through multiple routes, control of environment is laborious, actual dose received is difficult to calculate, and the cost is higher (Phalen *et al.* 1984; Dorato 1990; Drew 1990b).

In addition to the treatment system, the type of airflow within the chamber is a major determining factor of design. Chambers can be run under static or dynamic conditions. In static exposure systems gas is introduced as a batch, then mixing occurs and the air is not replenished. These types of systems are limited by oxygen depletion, carbon dioxide and water vapour accumulation, and loss of the test agent. Dynamic

systems in contrast continually replace chamber air and test materials (Silver 1946; Drew and Laskin 1973; Drew 1982). The latter is the more commonly used system in modern inhalation exposures for obvious reasons. Specific details of the individual chambers and environmental conditions used in this research will be described in subsequent sections.

Study design is the third major component of any inhalation evaluation. The experimental design should evaluate both local pulmonary and systemic effects of the chemical of interest. Exposure may be acute (1-4 hrs), acute multidose (1-4 hrs/day, 14 days), subchronic (1-4 hrs/day, 5-7 days/wk, 90-180 days), or chronic (1-23 hrs/day, 5-7 days/wk, 52-104 wks) and would ideally include a variety of species (Dorato 1990). Inhalation effects have been studied in rats, mice, hamsters, guinea pigs, cats, dogs, pigs rabbits, monkeys, and donkeys (Phalen *et al.* 1984).

1.7.1 Structural Components

This section deals with the basic physical components of an environmental inhalation chamber, gas generation system, construction materials, and general considerations.

1.7.1.1 Chamber Design

An inhalation chamber in its simplest form consists of a test air generation system, inlet for test air, air filtration system, apparati for temperature and humidity control, airflow monitoring and maintenance system, sampling port, animal exposure holding chambers, and an exhaust system. The size, shape, and volumes of systems vary greatly depending on the species exposed, treatment system, test chemical, number of animals, and duration of study (Macfarland 1983).

1.7.1.2 Test Atmosphere Generation

Generation of the contaminant air is often viewed as the single most important component of any inhalation study (Campbell 1976b). If the atmosphere is improperly generated the dose will not be uniform throughout the system and over the dose period making results difficult to interpret. Many systems are available depending on the physical and chemical properties of the contaminant. Gases and vapours are traditionally produced in ppm or mg/m^3 quantities by a vapour generator or bubbler (Hinners *et al.* 1968a). Once the gas is produced it is diluted and released to the chamber in a steady flow of clean, moist air. Constant monitoring is required to ensure the same concentration is released throughout the exposure duration (Drew 1990b). The most simple and reliable technique is the use of premixed gas cylinders (Campbell 1976a). The associated cost is higher but neither constant sampling nor purifying steps are required. Aerosols are much more difficult to produce than vapour because of the need to generate and characterize specific particle sizes. The current study is concerned only with gas generation hence aerosols will not be discussed further.

1.7.1.3 Construction Materials

Various materials have been used in construction since the inception of inhalation studies. The internal composition of the chamber is a major concern as it may act to lower the concentration by adsorption, absorption, and/or chemical reaction (Silver and Arsenal 1946).

The preferred metal for construction is stainless steel. In the presence of most chemicals, with the exception of chlorides, it is rust resistant and will not erode (Hinners *et al.* 1968b). The addition of 12% chromium produces this rust proof property. In

contrast galvanized steel is dipped in zinc, and will rust if submerged for extended periods of time. The associated costs and fabrication are much higher for stainless than galvanized steel. Galvanized steel is appropriate only where short duration, low moisture content, and suitable test chemicals are used (Hinners *et al.* 1968b).

Glass, as well as lacquer and enamel lined surfaces are inert and avoid significant loss of test chemicals (Silver and Arsenal 1946). However they require high maintenance and are not used in modern studies.

Gas conduction lines made of rubber or cellulose acetate will absorb a significant amount of chemical and should be avoided (Silver and Arsenal 1946). The preferred tubing is copper pipe (Praxair internal communications), connections of stainless steel or copper, and tubing composed of Polytetrafluoroethylene (PTFE). PTFE is a synthetic polymer with an extremely low coefficient of friction. It is very non-reactive, and so is often used in containers and pipe work for reactive and corrosive chemicals (Cole-Parmer internal communications). Polyvinyl chloride (PVC) is an inexpensive polymer that can also be used in construction of chambers. A study of benzene and toluene contained in PVC tubes showed minimal adsorption and absorption losses (Shahalam *et al.* 1997).

1.7.2 Operational Characteristics

The air within an inhalation chamber can produce deleterious effects in addition to the chemical being tested. To distinguish effects of the chemical vs. improper environmental conditions there are basic concepts, which must be considered, and optimum standards that must be established.

Air quality, temperature, and relative humidity are a function of flow rate, chamber volume, animal loading, and exposure duration. The individual effects of each

of these components will not be discussed here, only the standards, optimal conditions, and effects of deviations will be considered.

1.7.2.1 Air

An air change occurs when the volume of air equal to the volume of the chamber has passed through the chamber (Campbell 1976b). This ensures the animals within the chamber are being exposed to new air with the desired chemical concentration as well as proper oxygen content in order to meet metabolic demands. If the air changes are inadequate CO_2 or ammonia may increase or oxygen content may decrease to a toxic level. The number of air changes required for a dynamic flow chamber is 12 per hour (OECD 1981).

1.7.2.2 Temperature

Thermoregulation is controlled by homeostatic mechanisms in free living animals. This regulation becomes much more difficult in confined, closed environments. When animals are in fixed volume of space their metabolic heat production may increase the chamber temperature (Macfarland 1983). In a dynamic chamber, this problem is easily avoided if the incoming air is kept a few degrees below ambient (MacFarland 1983) or by using stainless steel for chamber walls which will transfer heat (Drew 1990b). The allowable temperature depends on the species being tested. Birds for example have a higher body temperature (40 C) than rodents (38 C), so chamber temperatures can rise to 26 +/-2 C (Bartholomew and Cade 1957) rather than 24 +/-2 degrees Celsius (OEHA). The temperature control should be established before starting a study. Hyperthermic conditions may adversely affect heart rate, respiratory rate, oxygen consumption, and enzymatic reaction rates (Randall 1943).

1.7.2.3 Relative Humidity

Relative humidity (RH) and temperature play a crucial role in the response of an animal to heat induced stress. Evaporative cooling is an important mechanism for temperature regulation in birds. It is driven by the gradient between body surface and air water vapour density (Marder and Arad 1989). If the RH is not optimal with respect to the test species hyperventilation may occur resulting in respiratory alkalosis, as is the case for birds (Marder and Arad 1989). The optimal RH for physiological conditions in birds is 60-65% (Yahav 2000).

1.8 Research Objectives and Hypothesis

There were two primary objectives addressed by this study.

1) Design and build a dynamic flow environmental inhalation chamber capable of exposing American kestrels to a binary mixture of benzene and toluene.

2) Evaluate the toxicological effects of exposure to rodent derived LOAELs and environmentally relevant levels of benzene and toluene in American kestrels by measuring the following biological effects/responses in exposed kestrels:

a) Cytochrome P450 1A1/2, 2B, and 2E
b) Antibody mediated immune response
c) Integrated immune response (T lymphocytes, B lymphocytes, and macrophages).
d) Plasma levels of triiodothyronine (T3) and thyroxine (T4).
e) Alterations in plasma retinol and liver retinyl palmitate levels.
f) Bone marrow erythroid and granuloid cell populations.
g) Complete blood cell count (polychromatophils, granulocytes, and lymphocytes).
h) Specific behaviours in response to a predator.

Null Hypothesis (Ho)

Inhalation exposure of American kestrels to benzene and toluene will result in greater immunological, haematological, endocrinological, neurological, and enzyme activity effects observed in mammalian studies using similar concentrations.

CHAPTER 2

THE DESIGN, CONSTRUCTION, AND OPERATION OF A WHOLE BODY INHALATION CHAMBER FOR USE IN AVIAN TOXICITY STUDIES.

2.1 Introduction

Environmental risk assessments are broadening to include evaluations of avian species exposed to gaseous and particulate materials (Mineau 2002; Irvine 2004; Carmalt 2005). Since the avian respiratory tract is fundamentally different from a rodent respiratory tract, the effects of gaseous materials on birds cannot validly be extrapolated from rodent exposure studies (Briant and Driver 1992). Over the past decades birds, especially poultry and waterfowl, have been used as models for inhalation studies and routes of vaccine administration (Fletcher *et al.* 1976; Eidson and Kleven 1976a; Eidson and Kleven 1976b; Myers and Arp 1987). Very few studies, (Weeks *et al.* 1977) have included exposing birds within inhalation chambers.

Birds are found in all environments inhabited by humans and can be used as highly sensitive monitors of air quality (Brown *et al.* 1997). To use birds as monitors of air quality or to study the effects of inhaled toxicants, exposure must occur in a controlled environment and results can then be compared to what is occurring in nature. In 1992 Briant and Driver designed a 'head only' avian inhalation exposure chamber for larger birds such as chickens and ducks that was targeted to ensure only respiratory tract exposure. This chamber required the bird to be placed on its sternum on a platform with a rubber seal fitted around its neck. This design would be adequate for domestic chickens,

turkey, and other fowl with long necks but would not be feasible for smaller passerines or falconiformes with short necks and tracheas (Brown *et al.* 1997). The model could however be modified into a whole body exposure chamber and used for exposing smaller avian species.

The basic concept of an inhalation chamber is straightforward. Animals are placed in a closed confined space while air containing the chemical(s) of interest is uniformly passed over them at a fixed flow rate and then subsequently released to an air filtration or scavenging system for removal of the chemical(s). These systems have varied in volume, shape (cylindrical or cuboidal), composition (wood, glass, or metal), exposure type (whole body or partial), and species exposed (rodents, dogs, guinea pigs, rabbits, and pigs) (Silver and Arsenal 1946; Hinners *et al.* 1968b; Drew and Laskin 1973; Snellings 1990).

Based on current literature it appeared that an inhalation chamber specifically designed for exposing small birds to air pollutants did not exist. Therefore one was designed and constructed to expose American kestrels (*Falco* sparverius) to two well studied air toxicants, benzene and toluene.

For the current project a whole body, dynamic flow, fixed rate inhalation chamber with an air purification system capable of dosing 12 American kestrels was constructed.

2.2 Materials and Methods

2.2.1 Structural Components

All measurements are outer diameters unless otherwise stated. A diagram of the avian inhalation chamber is presented in Figure 2-7 and a picture in Figure 2-8. For a complete description of all piping, tubing, connections, part sizes, materials, and suppliers as well as a thorough description of all procedures and techniques used to construct the inhalation chamber see Appendix A.

2.2.1.1 Gas Generation

Praxair Distribution produced benzene and toluene gas mixtures for low and high dose exposure groups under order number 637748-00 (Geismer, Los Angeles, CA, USA). High pressure aluminum cylinders with a 4.12m^3 capacity were gravimetrically filled with a breathing grade air carrier gas and a high dose - 10 ppm benzene, 80 ppm toluene mixture (Al BZ10MT1C-AS), or a low dose - 0.1 ppm benzene, 0.8 ppm toluene mixture (Al BZ0.1MT1C-AS). Mixtures were verified by the supplier to +/- 5% using gas chromatography with flame ionization detection (GC-FID). Ultra high-pressure steel cylinders with an internal volume of 6.43 m^3 containing breathing grade air (AI BR-K) were purchased from Praxair (Saskatoon, SK (2005) and St Laurent, PQ (2006) Canada) for control group exposures.

2.2.1.2 Inhalation Chamber Component Description

The avian inhalation chamber was comprised of a gas generation apparatus, an inlet system for test compounds, gas distribution area, animal holding chambers, and an exhaust system. All welded parts were manufactured by Journeymen welders (A&B Quality Welding Torch and Prop Repair, Dalmeny, SK, Canada, Medallion Pipe Supply

Company, Saskatoon, SK, Canada, Bergen Industries, Drake, SK, Canada, TSL Mechanical, Lanigan, SK, Canada). Inlet piping, connections, and hose barbs were soft or hard ¼ to ½" copper pipe purchased from Canadian Tire, GreenLine, or General supply at the University of Saskatchewan, Saskatoon, SK, Canada. All connections were soldered and leak tested using soapy water and observation of bubble formation. Animal holding chambers, exhaust filter housing and connections were 3 or 4" polyvinyl chloride (PVC) pipes attached with silicone and non-volatile adhesives (Canadian Tire Saskatoon, SK, Canada). Exhaust lines were 3/8" diameter vinyl tubing (GreenLine, Saskatoon, SK, Canada).

The contaminant gas mixture (Section 2.2.1.1) in high-pressure gas cylinders contained the required concentrations of benzene and toluene. An Re 212 dual stage, chrome-plated, forged brass body five port configuration, with a 316 L stainless steel diaphragm regulator with a compressed gas association (CGA) 590 connection (PRX 212-3301-75-000, Praxair, Saskatoon, SK, Canada) released contaminated/test air to the inlet line (Figure 2-1). Breathing grade air was controlled by a CGA 346 Oxygen L-Tec regulator (150-540). The two gas lines (contaminated and clean) leading from the cylinders were controlled by separate ball valves which merged into a common line (Figure 2-2) that was attached to the inlet port of the 316 stainless steel 65-mm direct reading 0-10 LPM Gilmont flow meter (C-32014-17, Cole Parmer Canada Inc, Anjou, PC, Canada) (Figure 2-3). The inlet flow meter released either contaminated or clean air to a 350 cc Salter 7600 bubble humidifier (Medigas, Saskatoon, SK, Canada) connected to the exhaust port (Figure 2-3). The humidified air was then directed equally into two hexagons, which opened into twelve animal holding chambers (Figure 2-4). Expired and

unused test chemicals and air was exhausted from the animal holding chambers to a central unit which housed activated C-40 carbon (General Carbon, Paterson, N.J., USA) in a polypropylene filter bag (PO5P3-PR, The Filter Factory Inc., Yuma, AZ, USA) (Figure 2-6). The amount of activated carbon needed to adsorb any benzene and toluene in the exhausted air was determined using the formula in Equation 2-1[a, b] and the software readout provided by General Carbon (Paterson, N.J., USA) (Table 2-1).

Use per session = Use per hour (lbs) * dosing time **(2-1[a, b])**

Total C 40 usage = Use per session * total dosing time

[a1] Benzene 10 ppm
0.01 lbs / 24 hrs * 1.5 hrs * 28 days = 0.0175 lbs

[b1] Benzene 0.1 ppm
0.0001 lbs / 24 hrs * 1.5 hrs * 28 days = 0.000175 lbs

[a2] Toluene 80 ppm
0.02 lbs / 24 hrs * 1.5 hrs * 28 days = 0.035 lbs

[b2] Toluene 0.8 ppm
0.0002 lbs / 24 hrs * 1.5 hrs * 28 days = 0.00035 lbs

Year 1 [a1+b1] = 0.0525 lbs of activated C40 required for 24 hrs/day x 40 day runtime.
Year 2 [a2+b2+a1+b1] = 0.053025 lbs of activated C40 required for 24 hrs/day x 40 days runtime.

The flow rate was maintained by attaching the 0.2 CFM vacuum pump (001-78164-00, Cole Parmer Canada Inc., Anjou, PC, Canada) to the exhaust port of the outlet flow meter (C-32014-17, Cole Parmer Canada Inc, Anjou, PC, Canada), which pulled decontaminated air through the inlet port of the exhaust flow meter (Figure 2-6).

An adult male American kestrel is shown in the animal holding chamber prior to securing the lid with the exhaust line (Figure 2-5).

Contaminant	Concentration (ppm)	Molecular weight (g/mole)	S (g)	W (lbs)	Q (CFM)	Life expectancy Hrs	Life expectancy Days	Use / day (lbs)	Use / study (lbs)
Benzene	10	78.12	0.0999	1	0.177	5580	233	0.01	0.0175[a1]
Benzene	0.1	78.12	0.0999	1	0.177	558029	23251	0.0001	0.000175[b1]
Toluene	80	92.14	0.2737	1	0.177	1349	56	0.02	0.035[a2]
Toluene	0.8	92.14	0.2737	1	0.177	134889	5620	0.0002	0.00035[b2]

S = capacity of C40 to bind benzene or toluene, W = lbs of C40 required, Q = flow rate (CFM)

Table 2-1 Read out from General Carbon software (General Carbon, Paterson, N.J., USA) explaining the total amount of activated C-40 Carbon required to adsorb all residual benzene and toluene used in a 27 or 28 day inhalation exposure study with American kestrels.

Common volume conversions used during inhalation chamber construction and design **(2-2)**

1 in^3 = 16.387 cm^3 = 0.00001639 m^3 = 0.0164 L
1 LPM = 0.0353 CFM
1 g = 1 ml = 0.001 L

Figure 2-1 Re 212 dual stage chrome-plated forged brass body five port configuration, 316 L stainless steel diaphragm regulator with a compressed gas association (CGA) 590 connection supplied by Praxair (PRX 212-3301-75-000, Praxair, Saskatoon, SK, Canada) attached to ¼" compression X 3/8" hose barb brass fittings for control of the benzene toluene gas mixture release.

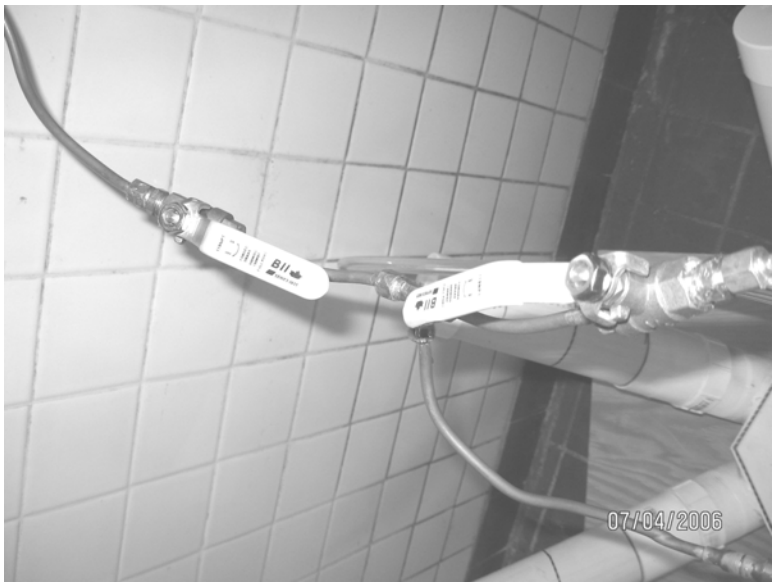

Figure 2-2 Separate ball valves for contaminated (left) and control (right) gas control soldered to a compression T exiting as a single ¼" soft Cu line that merges at the inlet port of the 316 stainless steel 65-mm direct reading 0-10 LPM Gilmont flow meter (C-32014-17, Cole Parmer Canada Inc, Anjou, PC, Canada).

Figure 2-3 Inlet port of the 316 stainless steel 65-mm direct reading 0-10 LPM Gilmont flow meter solid arrow (C-32014-17, Cole Parmer Canada Inc, Anjou, PC, Canada) attached to the gas feed system by ½" Cu soft pipe by a 1/8" FIP and at the top to the 350 cc Salter 7600 bubble humidifier hollow arrow (Medigas Saskatoon, SK, Canada) wing nut all exiting to the ½" hard Cu pipe leading to the animal chambers on the hexagon bases.

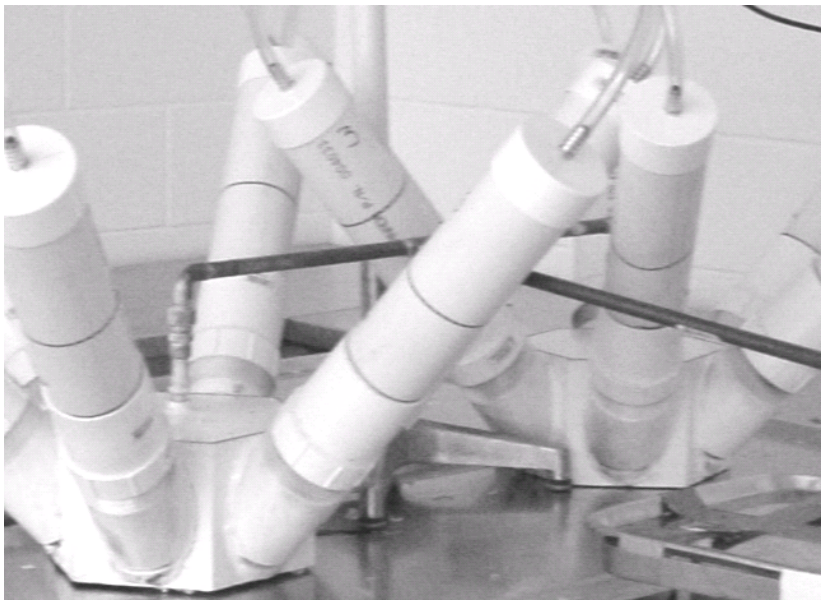

Figure 2-4 Bifurcation of ½" hard Cu gas lines that equally distribute incoming test gas to each of the two hexagons and subsequently to each of the 12 animal holding chambers

Figure 2-5 Top view of an adult male American kestrel placed in the animal holding chamber before the lid was secured.

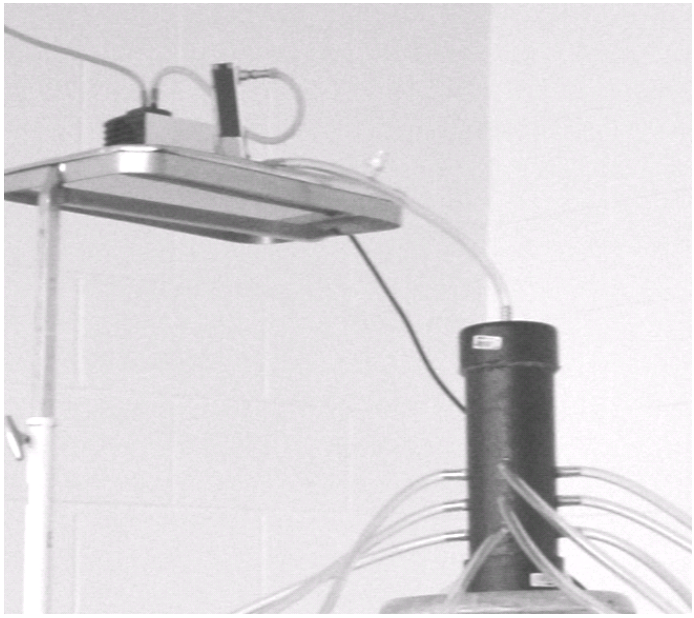

Figure 2-6. Pictured are 9 of the 12 individual holding chambers exhaust gas lines emptying into the black PVC filter housing unit (bottom). An exhaust line running to the bottom of the exhaust flow meter is attached to the 0.2 CFM vacuum pump (001-78164-00, Cole Parmer Canada Inc., Anjou, PC, Canada) that exits to a HEPA filtration system.

2.2.1.3 Volume

The volume of the current inhalation chamber was calculated using cylinder[a] and hexagon[b] volume formulas and has a total volume of 41 L or 2519.6 in^3 (Table 2-1).

Table 2-2 Total chamber and individual component volumes used in constructing the inhalation chamber used to expose American kestrels to benzene and toluene.

Component	Materials	Radius(r) (in)	Height(h) (in)	Volume (in^3)
Filter	PVC	2	15	188.5
Tubing inlet ¼"[a]	Copper	.125	49	2.41
Tubing inlet ½"[a]	Copper	.25	14	2.75
Flow meter inlet ½"[a]	Stainless Steel	.25	60	11.78
Holding chambers(12)[a]	PVC	1.5	18	1526.04
Exhaust from cylinder[a]	Vinyl	.1875	341	37.66
Exhaust tubing[a]	PTFE	.1875	15	0.02
Humidifier[a]	Plastic	1.25	5.25	25.8
Hexagon (2)*[b]	Steel	10	5	750
Total volume				2519.16

[a] Calculation of cylinder volume using formula $\pi r^2 h$
[b] Calculation of hexagon volume using formula $V = Bh$ or $V = 3ash$
r = radius, h = height, B = area of base, a = apothegm length, s = side length

2.2.1.4 Operation

The inhalation chamber in the current study had a constant 5 LPM input of test air from premixed gas cylinders that was measured by an input flow meter (Cole Parmer C-32014-17) in series before the animal chambers. The exhaust system included a 0.2 CFM vacuum pump (Cole Parmer, 78164-00) which pulled expired and unused test air through an activated C-40 charcoal filter which was monitored by an exhaust flow meter (Cole Parmer C-32014-17). Test air was continually introduced at 5 LPM and exhausted from the chamber at 5 LPM producing a dynamic horizontal flow system. Inlet and exhaust

flow meters were calibrated using a wet test meter (American wet test meters, Model AL-17, Elster, Canadian Meter Company Inc, Cambridge, ON, Canada).

2.2.1.5 Animal Load

Individual cylinders were designed to fit the body of a male American kestrel or a 110 gram bird. The length (8 ¾"– 10 ½"), width 2.6" and preferred vertical body position of kestrels were considered. The holding chamber radius was 1.5" which gave the birds ~ 0.4" of extra space. The animal load was 3.22% of the total volume of the system (Equation 2-3) (MacFarland 1983).

$$((W * N) / V) * 100\% = \text{animal load } \% \quad \textbf{(2-3)}$$

W = Average wt of Adult male American kestrel = 110 g = 0.110 L
V = Chamber volume = 41 L
N = Number of animals in chamber = 12 ((0.110 L * 12) / 41L) * 100% = 3.22%

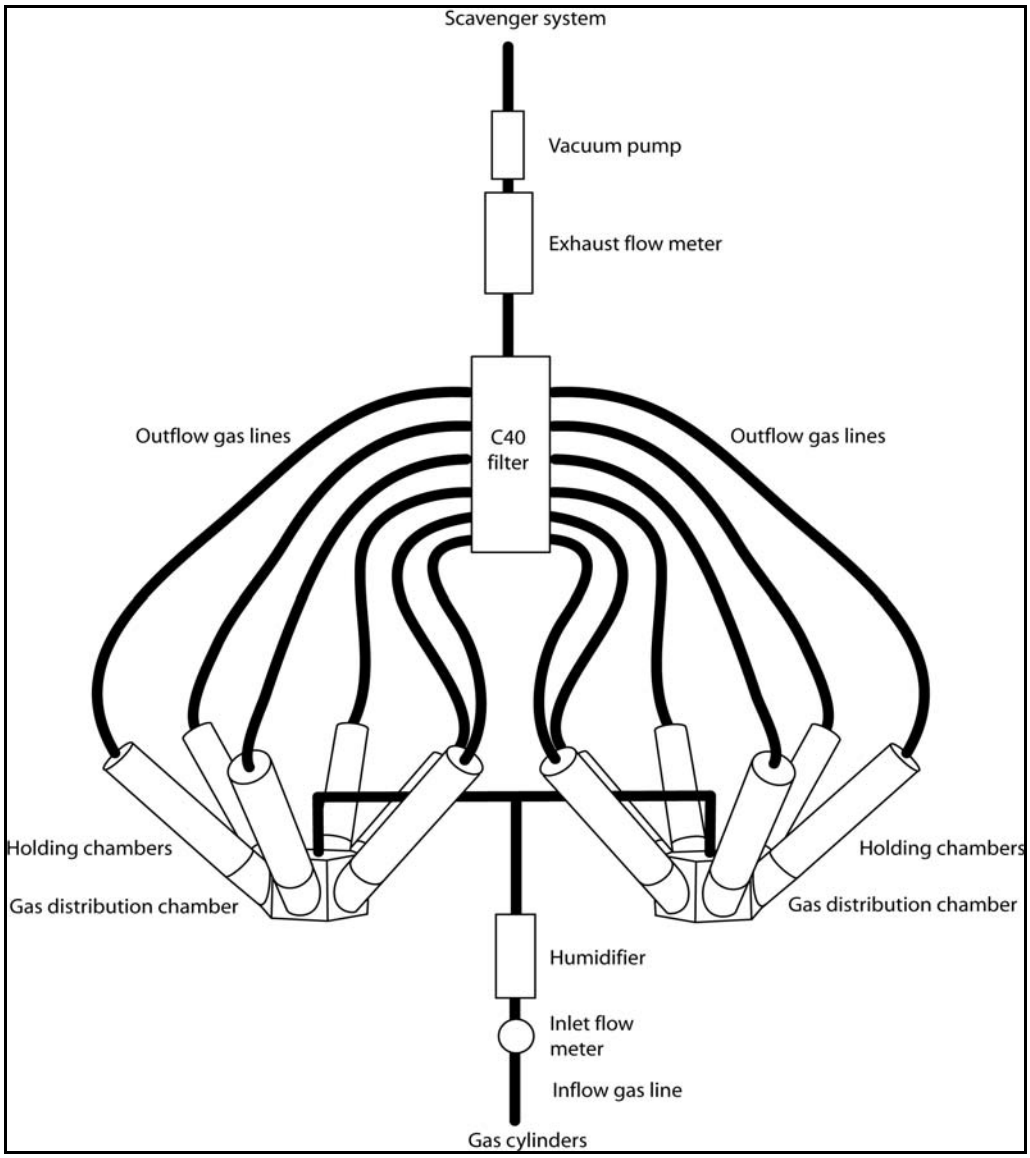

Figure 2-7 Diagram of the environmental inhalation chamber designed and constructed for exposing adult male American kestrels to a benzene and toluene gas mixture.

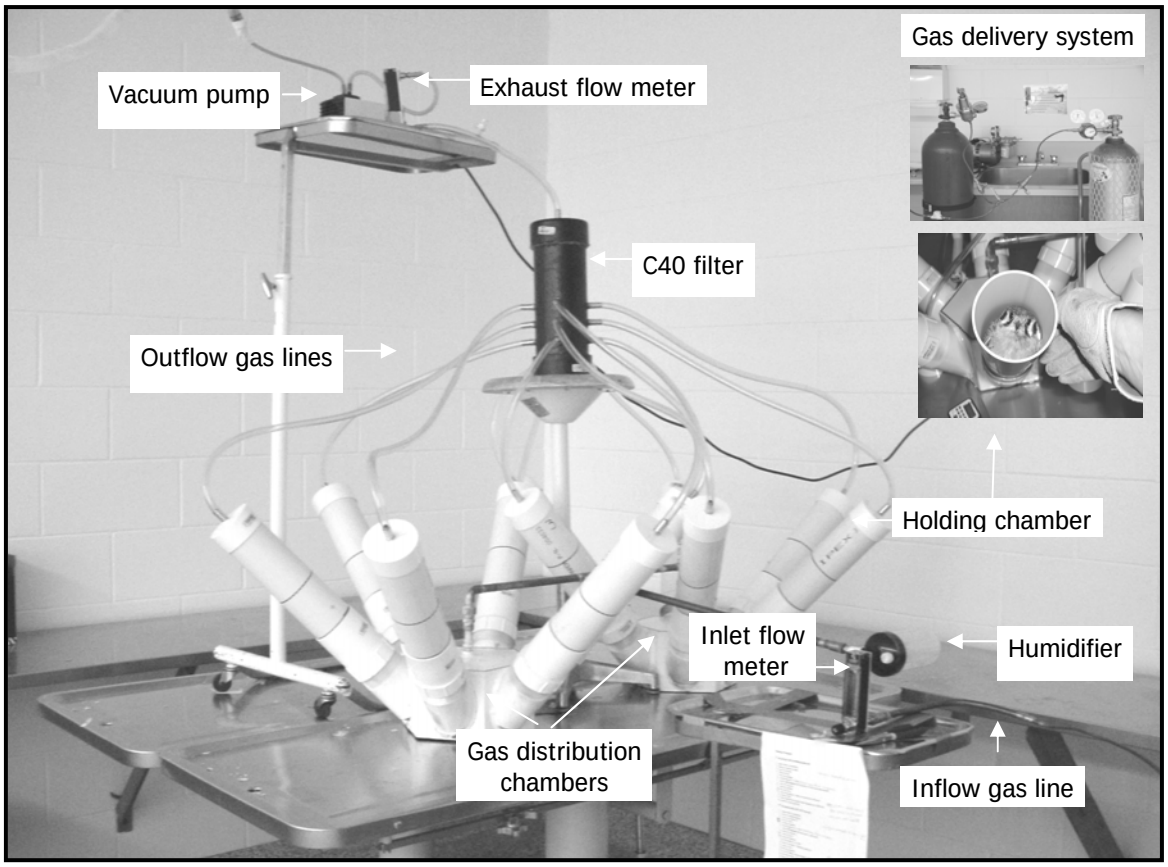

Figure 2-8 Photograph of the environmental inhalation chamber designed and constructed for exposing adult male American kestrels to a benzene and toluene gas mixture.

2.2.2 Operational Characteristics

2.2.2.1 Flow Rate

The calculated air requirement for proper respiration was 0.14362 LPM per American kestrel (Equation 2-4[a]) (Brown *et al.* 1997). In order to safely dose 12 birds and have 2 bifurcations in the system this number was recalculated and 3.45 LPM was required to ensure adequate oxygen supply within the chamber (Equation 2-4[b]). The flow rate was increased by 70% to 5 LPM or 0.177 CFM as an additional safety precaution (Equation 2-5) (Casarett and Doull's 2001).

Table 2-3 Air requirements for American kestrels based on respiratory rate, body mass, and respiratory tidal volume.

Variable	Calculation	Values
Resting ventilation	$V = 291.0\ 0(.110^{0.74})$	56.820 ml/min
Avian tracheal volume/dead space	$V_D = 3.724\ (0.110^{0.96})$	0.332 ml
Effective Ventilation	$V_P = V - V_D$	56.488 ml/min
Tidal volume resting (ml)	$V_T = 16.9\ (0.110^{1.05})$	1.67 ml
Tidal volume flight (ml)	$V_T = 27.8(0.110^{0.89})$	3.89 ml
Resting respiratory rate (RRR) (per min)		40 /min
Capture (Average) respiratory rate (CRR) (per min)		86/min

V = Ventilation (ml/min), V_p = Effective parabronchiolar ventilation (ml/min)
V_D = Avian tracheal volume (ml), V_T = Tidal volume (ml), M_b = Body mass (kg)

Resting V_T * CRR * 2 bifurcations * 12 cylinders = Flow rate ml/min **(2-4[a,b])**

[a]1.67 ml * 86 /min = 143.62 ml air per minute per bird

[b]143.62 ml per bird * 2 * 12 = 3446.88 ml/min

3.45 LPM American kestrel respiration requirements

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(3.45 LPM / 5 LPM) * 100% = 70% increase in flow rate **(2-5)**

2.2.2.2 Air Changeover Rate

The current study maintained a 5 - 5.5 LPM flow rate in a 41 L inhalation system which resulted in 7-8 air changes per hour (Equation 2-6, Table 2-4) (MacFarland 1983).

Flow rate (LPH) / System Volume (L) = number of air changes per hour **(2-6)**

300 LPH / 41 L = 7.3 changes/hr

Table 2-4. Flow rate in litres per minute or litres per hour required for air turnovers within the inhalation chamber. Calculations are based on the 41 L system designed for the current study.

Air changes (Hr)	Flow rate (LPH)	Flow rate (LPM)
12	492	8.2
11	451	7.5
10	410	6.8
9	369	6.2
8	**328**	**5.5**
7.3	**300**	**5**
7	287	4.8
6	246	4.1
5	205	3.4
4	164	2.7
3	123	2.0
2	82	1.3
1	41	7.0

2.2.2.3 Equilibration

The time required for gas released to reach equilibrium in the avian inhalation chamber was based on previous studies (Silver 1946). Following is the derivation of the calculation and the times required to reach 99% equilibrium (Equation 2-7) and the time required for the inhalation chamber used in 2005 and 2006 to reach 99% (Equation 2-8) and 55% equilibrium (Equation 2-9). The balance of equilibrium occurred quickly from this point, and 55% was considered adequate to start a 1½ hour exposure.

Concentration within the chamber assuming perfect mixing: **(2-7)**
$C = w/b\ (1-e^{-bt/a})$

Percent of desired concentration w/b in time t is:
$\% = 100\ (1-e^{-bt/a})$

Since only 99% equilibrium is possible:
$100\ (1-e^{-bt/a}) = 99$

Transformed into logarithm:
$e^{-bt/a} = 100-99/100 = 0.01$
$- bt/a = \ln 0.01 = 4.6052$

Therefore the time to reach 99% equilibrium is:

T_{99} (min) = 4.6052 a/b

C = concentration in mg/L at time t
w = mg of agent introduced / minute
a = volume of chamber (L)
b = Flow rate (LPM)
e = base of natural log, 2.7182
t = time (min)

T_{99} (min) = 4.6052 a/b **(2-8)**
T_{99} (min) = (4.6052) 41.28 liters / 5 LPM
T_{99} = 38.4 minutes

$e^{-bt/a}$ = 100% - 55% / 100% = 0.45 **(2-9)**
$- bt/a = \ln 0.45 = 0.7985$
T_{55} (min) = (.7985) 41.28 Liters / 5 LPM

T_{55} = 7 minutes

2.2.2.4 Relative Humidity

The average relative humidity (RH) in Saskatoon, Saskatchewan (test site) from May to August is 67%. The OECD recommends 30-70 to maintain proper breathing conditions for experimental animals. To saturate the test air a stem and diffuser 350 cc Salter 7600 bubble humidifier (Salter Labs, Arvin, CA, USA) (Medigas Saskatoon, SK, Canada) containing 100 ml of water was attached to the inlet flow meter. Equation 2-10 outlines the determination of water volume required (Manabe and Wetheral 1967).

Part 1. Calculating 100% Relative humidity **(2-10)**
$RH = P_1 / P_2 * 100\%$
P_1 / P_2 = mol water (18 grams) / mol air (29 mol / 22.9 L)
= 17.54 mmHg / (760 mmHg – 17.54 mmHg) * 18g/mol* 29 mol / 22.9 L * 1000 L

550.2 grams H_2O/m^3

Part 2. Calculating 67% Relative humidity
RH * Required RH =
= 550.2 g/m^3 * 0.67 RH

368.5 grams H_2O/m^3

Part 3. Calculating water requirements for 1.5 hr dose session
67 % RH g/m^3 * flow rate = H_2O required for 1.5 hours
= 368.5 g/m^3 * 0.177 m^3/h
= 65.22 g H_2O per hour

97.83 g or 97.83 ml of H_2O per dose session.

P_1 = actual vapour density / atmospheric pressure 760mm/Hg @ 20 C
Ps = saturation vapour density / water vapour pressure 17.54 mmHg @ 20 C
P_2 = vapour density - saturation vapour density

2.2.2.5 Temperature

The temperature within individual occupied cylinders was monitored for 70 minutes at 5 min intervals. The average temperature within the animal holding chamber was 25 C over a 70 minute dosing session with a range of 24-27 C. The data is shown below in Figure 2-9.

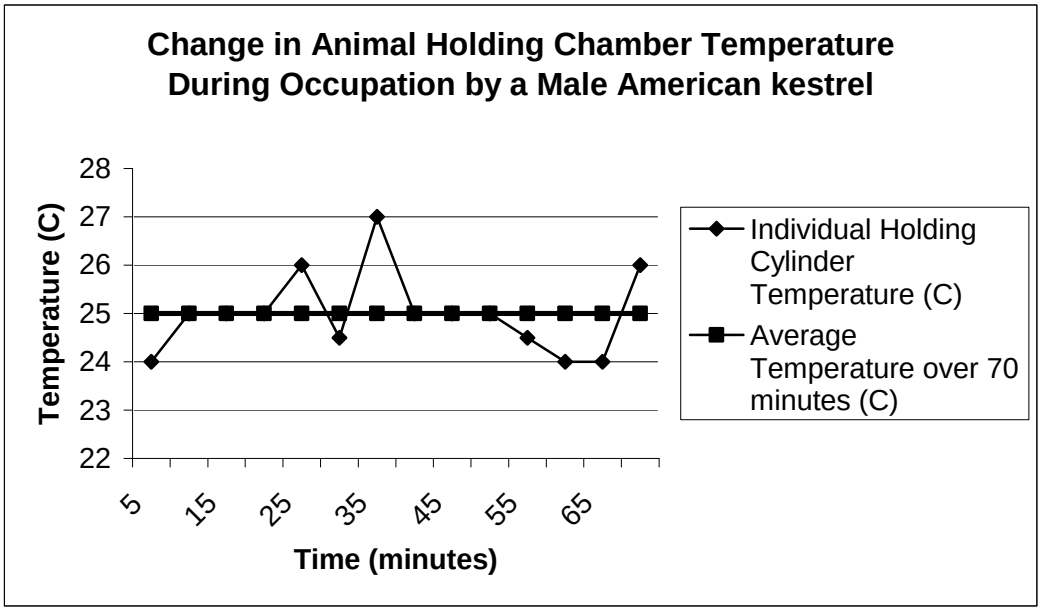

Figure 2-9 Change in temperature (C) in individual animal holding chambers over the 70 minute sampling period during occupation by adult male American kestrels.

2.3 Discussion

Table 2-5 outlines the recommended structural components and operational characteristics (Silver and Arsenal 1946; Hinners *et al.* 1968c; Phalen 1976; Drew 1982; MacFarland 1983; Macfarland 1983; Phalen *et al.* 1984; Snellings 1990; Drew 1990a; OECD 2005) and the variable counterparts in the chamber that was constructed and used to expose American kestrels to a benzene/toluene gas mixture.

Table 2-5 Guidelines and proposed variables for the construction and exposure of animals to inhalation toxicants.

Variable	Guidelines for animal exposures (mammal)	Proposed inhalation chamber (avian)
Gas Generation	bubbler, gas cylinder	gas cylinder
Construction Materials	glass, stainless steel, galvanized steel, teflon, inert plastics	galvanized steel, copper, polyvinyl chloride
Volume[a]	highly variable	41 Litres
Flow Rate[b]	highly variable	5 Litres per minute
Animal Load	Maximum 5% of total volume	3.22% of total volume
Air Changes	8 - 20 per hour	8 per hour
Equilibration	99% before dosing	55 % before dosing
Relative Humidity	30-70%	Calculated to 67%
Temperature	Maximum 26.7 C	Average 25 C

[a] function of animal load
[b] function of animal breathing requirements, volume of system

Gas Generation

The air quality standards requiring any impurities to be filtered (Drew 1982) before exposing test animals was not applicable to this study which was based upon laboratory generated, premixed, breathable grade air, benzene, and toluene that were certified to be at least 95% pure by GC-FID (Geismer, Los Angeles, CA, USA).

Monitoring of the exposure atmosphere in the chamber ensures that generated test air is being delivered at the predetermined concentration (Drew and Laskin 1973;

Snellings 1990; Drew 1990a). Air was sampled from the chambers once per dosing session in both contaminant exposed and control groups in 2005. Samples were drawn out of the inlet gas line by a gas tight syringe (Varian Inc, Mississauga, ON, Canada) and analyzed by GC-FID to confirm the presence or absence of test compounds. Daily records show no deviation from desired concentrations of benzene and toluene mixture in the breathable grade control group air in 2005, so further daily testing was not continued in 2006.

Gases such as benzene and toluene can be generated by either flow dilution or liquid dilution. Flow dilution requires the metering of contaminant gas and breathing grade air that are subsequently mixed and released into the chamber. Liquid dilution systems are more complex and are required when the contaminant is liquid at room temperature. In order to create a gas, the liquid must be heated, the volatilized product captured then released into an air stream entering the chamber. To avoid complications associated with generating the gas mixtures on site, a more costly but secure route was chosen; the gases were supplied in high-pressure gas cylinders by a commercial company set up to do this. Cylinders were gravimetrically filled with a mixture of benzene and toluene that was consistently released at the required ppm until the tank pressure fell below 50 kpa or ~10 psi. Gas generation was simplified by using premixed cylinders negating the need to generate, mix with filtered air, measure and then release to the chamber.

Construction Materials

Material selection is an extremely important feature in designing an inhalation chamber. The material from which the gas generation, inlet system, gas distribution and

animal chambers, and exhaust system are made, will determine the amount of test chemical lost due to adsorption and absorption. The least adherent materials with respect to gases are glass, Teflon®, stainless steel, and non-stainless steel. These materials are expensive and difficult to work with but are considered the primary choices unless chloride or sulfur containing compounds are being tested (Hinners *et al.* 1968a). Non-stainless steel is cheaper but rusting is a major concern when moisture build-up and defecation/urination may occur within the chamber. Considering the short duration of the exposures in this study and lack of defecation while birds occupied the chamber, the probability of rust was deemed negligible.

Polyvinyl Chloride (PVC) was used for the animal holding chambers and filter housing because it has low adsorption and absorption of benzene and toluene (Shahalam *et al.* 1997). Vinyl tubing was used for the exhaust gas lines for the same reasons. Plastics tend to age rapidly and build areas of high static charge which are undesirable traits but do not limit the use of such materials (Dorato 1990).

Copper pipe was used for the inlet gas lines mainly because of ease of handling and because it is resistant to erosion and high pressure, Cu pipe is used in almost all plumbing. These qualities are also very important in the materials used for inhalation chambers.

Inhalation Chamber Components

The whole body chamber design was the only feasible option for American kestrels. A 'nose only' exposure would have been impractical considering the shape of the head of the American kestrel. Also restraint would have been required to restrain the head and pin down the wings, which would have compromised respiration, and would be

considered inhumane by Animal Care standards. An endotracheal tube that would allow for a more controlled exposure would require anaesthesia of the birds, another unacceptable stressor. The inhalation chamber patented by Briant and Driver for birds was also not usable for short-necked, vertical perching kestrels, having been designed for larger birds with different body conformation such as chicken and ducks (Briant and Driver 1992).

The chemicals of interest, benzene and toluene, have established toxicities associated with inhalation in mammals (ATSDR 2000; ATSDR 2006). Therefore the experimental air had to be purified before being release to the environment. Activated carbon 40 derived from bitumen was purchased as 4 mm pellet from General Carbon. The high adsorptive activity and huge surface area make it ideal for most vapor phase applications. Activated C-40 specifically binds VOCs and is highly efficient at low pressures making it an ideal filtering agent for the current study (Matranga *et al.* 1992). The amount of activated C-40 required to remove benzene and toluene from the test atmosphere after a 27 day exposure period was 0.0525 lbs. The polypropylene filter held 1 lb of C-40, the ppbRAE readings (Appendix A Table 1) showed a gas input of 90 ppm mixed VOCs and a maximum output of 0.7475 ppm VOCs indicating the C-40 filter was eliminating 99.17% of all VOCs entering the chamber. The Department of Health and Safety (DHSE) at the University of Saskatchewan confirmed this to be acceptable for atmospheric release.

Volume and Flow rate

The volume of the inhalation chamber influences the flow rate, air changes per hour, and amount of chemical needed. A greater chamber volume means more test

chemical, longer equilibration times, and higher flow rates will be required. A smaller system decreases these amounts but could limit the number of animals that could be exposed at one time. The flow rate must ensure adequate air delivery for animals with a higher than normal respiratory rate to ensure we are observing the effects from the test compounds rather than from hypoxic conditions. The airflow was monitored at the inlet and outlet flow meters, which were constructed of inert materials. Breathing requirements of 12 American kestrels plus the minute volume ensured that we could use a low flow rate (Daniels and Duke 1980; Brown *et al.* 1997). The volume is minimal compared to other inhalation chambers (41 L vs. 270 L) (Hinners *et al.* 1968a; Drew and Laskin 1973; Saito *et al.* 2000) and the required flow rate is much lower (5 LPM vs. 300) LPM thereby minimizing the amount of test gas required.

Animal Load and Airflow

The animal load is a measure of the relative volume of the inhalation system occupied by the test animals. The load was kept below 5% of the total system volume, which is the maximum loading capacity to ensure proper temperature regulation and maintenance of the test compound concentrations. By occupying only 3.22% the load would not significantly increase the temperature or decrease the concentration of the test chemical in the atmosphere due to animal surface effects (Drew and Laskin 1973).

The number of air changes per hour varies from 8 to 60 (Drew 1982). This is a function of the animal load, study duration, and control of temperature, oxygen and carbon dioxide levels (Drew and Laskin 1973). We exposed 12 birds for 1-11/2 hours, 5 days a week. Based upon other studies with similar designs 8 air changes per hour were sufficient.

The OECD standard requires 12 air changes per hour for a 6 hour dose session (OECD 1981). This makes the 8 air changes per hour seem inadequate to meet oxygen demands and maintain optimal temperatures for the exposed birds. However, if we consider three important features of the study and chamber design this was not a problem. First, the flow rate was calculated based on the specific oxygen requirements of male American kestrels and supplied 70% more than the calculated requirement of 142 ml/min for each bird (Table 2-2, Equation 2-2). Second, the chamber temperature with the bird inside was monitored and the average 25 C indicated adequate airflow for thermoregulation (Table 2-3), and third, if the animal load does not exceed 5% of the total chamber volume, this will decrease the required number of air changes. The calculated oxygen requirements, increased flow rate, consistent chamber temperature, and the low animal loading, supplied enough evidence that the 7-8 air changes per hour would have no adverse affects on test subjects.

Equilibration

The duration of daily dosing was based upon a combination of rodent inhalation studies, minimizing stress to the birds, and providing an environmentally meaningful total dose. An important factor for calculating daily dosing time was the time required for the system to equilibrate. This ensured that all birds were receiving the same concentration of chemicals when dosing time started, and that the system was cleared of any chemicals when the dosing time was complete or when control birds were placed into the chambers. Previous studies have used a 99% equilibrium standard before official dosing time begins. Due to an error in calculation the dose time began after 7 minutes when chamber equilibrium was at 55% rather than the 99% equilibration, which occurred

at 38 minutes (Equation 2-9). If we consider that 99% saturation of the chamber was achieved within half an hour of the dosing start time, we know the birds were exposed to the compounds of interest, at the doses of interest for about 1 hour per day, which made the study somewhat more conservative than intended. This makes any effects that were detected, even more significant than previously thought. Follow-up studies should however reach 99% equilibrium before the start of dosing.

Relative Humidity

Gas cylinders used in the pilot study contained less than 1% moisture (Praxair, Internal communication. 2005) which was inadequate for birds (Brown *et al.* 1997). To achieve 67% RH a bubble humidifier with 100 ml of water was used during each exposure. The birds were not indicating respiratory distress when removed from the chamber. In future studies I would choose a different method to control the RH for two main reasons. First, not all of the water in the bubble humidifier was taken up into the air entering the system and secondly, the RH of the chamber air was not measured to validate the bubblers' efficacy.

Temperature

Thermoregulation is controlled by homeostatic mechanisms in mammals and birds. This regulation becomes more difficult in closed, confined environments, where metabolic heat production may increase the ambient temperature. In a dynamic chamber this problem is easily avoided if the incoming air is a few degrees below ambient or by using stainless steel for chamber walls which will readily transfer heat (MacFarland 1983). The room temperatures where the chamber was used were 18 and 21 C. The temperature in the bird-inhabited chambers was 25 C resulting in heat transfer out

through the walls. The PVC pipe of the holding chambers would be poor at temperature transfer compared to stainless steel, but this did not to pose a problem. American kestrels for which the chamber was designed, have a body temperature ~ 40.5 C, making 25 C quite acceptable. By keeping the temperature moderately low, heart rate, respiratory rate, oxygen consumption, and enzymatic reaction rates (Randall 1943) would not be negatively affected in the test birds from a thermoregulatory aspect.

2.4 Conclusion

The inhalation chamber described in this chapter was successfully used to expose adult male American kestrels to premixed gas mixtures of benzene and toluene. The chamber was designed using basic principals of structural design and operational characteristics from early researchers such as Silver, Drew, Laskin, and Campbell. Following the basic principals and considering the physiological requirements of birds a safe and practical inhalation chamber was built to allow exposure of wild birds to air pollutants of concern.

By designing a system specifically for a bird model that exposes non-mammalian test species we can generate data that is specific for a sensitive family of animals, Aves. This allows us to gain insight into the mechanism of action of airborne pollutants in non-mammalian models. The bird has long been used as an early indicator of poor air quality in the mining industry. This inhalation chamber allows us to expand on this concept and study in a controlled laboratory atmosphere the neglected area of inhalation toxicity of xenobiotics in wild birds.

CHAPTER 3

EFFECTS OF INHALATION EXPOSURE TO BENZENE AND TOLUENE ON THE HUMORAL, CELL- MEDIATED, AND INNATE IMMUNE RESPONSES AND ASSOCIATED ENDOCRINE SYSTEMS IN AMERICAN KESTRELS.

3.1 Introduction

Immunotoxicology is the study of the adverse effects on the immune system from exposure to environmental chemicals, drugs, or biological materials (Luster and Blank 1987). Several classes of chemicals (halogenated hydrocarbons, PAHs, organochlorine compounds, metals, PCBs, brominated diphenyl ethers, and pulp and paper effluents) have well-associated immunotoxic effects in wildlife (Smits *et al.* 1996; Fox *et al.* 1998; Smits and Bortolotti 2001; Smits *et al.* 2002; Fairbrother *et al.* 2004; Fernie *et al.* 2005a).

Chemically VOCs in flare gas are unsaturated cyclic (aromatic) hydrocarbons that contain one or more benzene rings (ATSDR 2005). Benzene, toluene, ethylbenzene, and xylene (BTEX) are often used as representatives of this group. Benzene and toluene are hazardous because of their inherent toxicities, wide use in industry, and high volume of production which leads to substantial environmental releases (Robinson *et al.* 1997). They have therefore been selected as the chemicals of concern for this study addressing the toxic effects of airborne contaminants on wild birds.

In order to have a fully functioning protective immune system, all compartments must be regulated properly and in communication with one another. Proper communication is controlled by cytokines that are produced by the cells of both arms of

the defense system. Hormones such as thyroxine (T4) and triiodothyronine (T3) and vitamin A derivatives, retinol and retinol palmitate (RP) also play important roles in modulating the immune response. Thyroid hormones (THs) are responsible for growth, differentiation, cellular metabolism, and overall hormonal balance within an individual (Rolland 2000), while vitamin A plays a role in antibody production, T lymphocyte responses, and phagocytic reactions, cellular maturation and differentiation (Friedman and Sklan 1997). Low levels of TH have been shown to exacerbate immunomodulation (Smits *et al.* 2002; Fairbrother *et al.* 2004; Decuypere *et al.* 2005) and the status of plasma retinol can affect TH concentrations as they share a common carrier protein, transthyretin (TTR) (Brouwer *et al.* 1990; Rolland 2000). Any chemical that alters TH or retinoid levels in the plasma can lead to an increase or decrease of thyroxine or retinol in circulation which in turn can alter tissue levels and cellular messaging leading to improper immune function, cellular differentiation and/or a loss of hormonal regulation (Rolland 2000).

Benzene is a well-known immunotoxicant that targets maturing B and T lymphocyte precursors in the bone marrow. The phenolic metabolites from benzene's biotransformation enter a reduction-oxidation cycle and produce reactive oxygen species (ROS) that bind cellular macromolecules causing immune cell death in the bone marrow (Rozen *et al.* 1984a; Snyder 2004; ATSDR 2006). The lowest observable adverse effect levels (LOAELs) for these immune effects in mice is reported at 10 ppm (Rozen *et al.* 1984b). When animals are co-exposed to toluene and benzene, the adverse immune effects are diminished. Toluene is thought to be protective against benzene related immunotoxicity by preferentially binding CYP2E1, the biotransformation enzyme

responsible for benzene metabolism, thereby decreasing the toxic metabolite load in the body (Purcell *et al.* 1990a).

The National Institute of Environmental Health (NIEH) and National Toxicology Program (U.S.Public Health Service 2005) have designed a battery of Tier I and II tests that can be used to determine the immunotoxic potential of chemicals (Luster et al. 1992). The animal models for these tests have historically been mammalian (Luster *et al.* 1988), resulting in the need for laboratory/researcher specific validation of tests in alternative wildlife species (Smits *et al.* 1999; Smits and Williams 1999). Tier I includes, hematology, organ morphometrics, histological examination of immune organs, and lymphocyte blastogenesis potential and generally involve the loss of animal life. Tier II involves, quantification of B and T lymphocyte numbers, antibody levels, delayed type hypersensitivity, and host resistance assays, in an effort to determine more sub-lethal effects while preserving life. A good assessment of the overall immune function of an exposed individual or population will implement tests from both Tier I and II (Luster *et al.* 1992; Luster *et al.* 1994).

Taking into consideration the high concentration and immunotoxic potential of benzene and other halogenated hydrocarbons such as toluene present in ambient air samples on the prairie and boreal plain regions of Saskatchewan and Alberta (Waldner *et al.* 2001) and the prevelance of raptors in these areas (Kirk and Hyslop 1998; Hoffman and Smith 2003), a laboratory-based inhalation exposure study was designed to test a sensitive bird species.

The American kestrel (*Falco sparverius*) was chosen as a study species because it has a respiratory tract unique to birds, which results in greater absorption and metabolism

of air contaminants by the lung compared to mammals (Brown *et al.* 1997). The kestrel serves as a valid and useful sentinel species owing to its small size, non-endangered status, relatively easy maintenance in captivity, and its value as a model for other larger prairie-inhabiting falcons (Wiemeyer and Lincer 1987).

The objectives of this study were to determine the effects of benzene and toluene on the humoral (antibody production), cell-mediated (delayed type hypersensitivity), and innate (granulocyte populations) immune systems of American kestrels and the possible thyroid hormone and vitamin A alterations.

The proposed study assessed the overall function of the immune system of American kestrels after exposure to environmentally relevant levels of benzene (0.1 ppm) and toluene (0.8 ppm) as well as to rodent LOAEL levels of benzene (10 ppm) and toluene (80 ppm) administered via inhalation.

3.2 Materials and Methods

3.2.1 Inhalation Exposure of American kestrels to Benzene and Toluene

3.2.1.1 Animals and Housing

Year 1

In May 2005, 23 male American kestrels were captured using Bal-chatri traps in a 100 km radius of Prince Albert, Saskatchewan (SK), Canada. The birds were transported to a flight barn 7 km from Saskatoon, SK, Canada where they were acclimated for 5 weeks before being transported to the Animal Care Unit (ACU) at the University of Saskatchewan (U of S) (Saskatoon, SK, Canada). Once at the ACU, kestrels were divided by weight into two dose groups; n=12 high dose group, n=11 control dose group. Treatment groups were housed separately in windowless rooms with shavings covering the floor, two equal height rope perches, a water bath, greenery in the form of tree branches, and three feeding stations. Each room was on a 12-hour light/dark cycle beginning at 0730. Feeding times were staggered at 1030 (control group) and 1230 (high group) based on dosing times to ensure that the crop was not full prior to daily exposures or blood sample collection. The diet consisted of 1½-day-old cockerel chicks per kestrel per day (Anstey Hatchery, Saskatoon, SK, Canada). Daily records were kept regarding number of chicks fed, time of feeding, water bath changes, bird removal/return times and mortality.

High and control dose groups were acclimated for two weeks before exposures began. Baseline mass and body body condition were recorded, and blood and feather samples were collected 1 week prior to dosing. Over the next 6 weeks of challenge exposures, birds were weighed weekly and bled after weeks 1, 2, 4, 5, and 6 (necropsy).

Year 2

In May 2006 32 adult male American kestrels with previous *in ovo* exposure to polybrominated diphenyl ethers (PBDEs) were selected from the breeding colony at the Avian Science and Conservation Centre (ASCC) of McGill University at Macdonald Campus (Ste Anne de Bellevue, PQ, Canada). Birds were divided into three dose groups (n=11 control, n=10 low dose, and n=11 high dose), placed in separate rooms for the duration of the study, and baseline blood samples and weights were taken on the isolation day before acclimation. Each room had screened windows, a feeding bench, water bath, 2 rope perches, and shavings covering the floor. Birds were weighed weekly and blood samples were collected on weeks 1, 4, 5 and 6 (necropsy).

Birds were fed as in dose year 1 (Section 3.2.2.1) except the third, high dose group was fed at 1430 for logistical reasons.

All blood samples were collected according to guidelines of the ACU at the University of Saskatchewan and for a WCVM-sponsored bird-bleeding workshop (Drs J. Smits, K. Machin, D. Parker, and C. Wheler). Approximately 1 ml of blood was collected from the jugular vein using 7.5% ethylenediamine tetraacetic acid (EDTA) (2005) (Sigma, Aldrich St. Louis, MO, USA) or Heparin Sodium- (2006) (Hepalean, Organon Teknika, Toronto, ON, Canada) coated syringes and 28 gauge needles. Samples were placed on ice in 1.5 ml eppendorf vials. No more than 1% of an individual's total body weight was collected. For each bird, two blood smear slides were made and the remaining blood was microcentrifuged (Brinkman instruments Eppendorf Microcentrifuge, 5415D, VWR International, Mississauga, ON) at 1500 rpm for 5 min. Plasma was then removed and placed in a -40 C freezer for future analysis. In accordance with Canadian Council on

Animal Care guidelines, all experiments were approved under University of Saskatchewan protocol number 20050025 and University of McGill protocol number 5236.

3.2.1.3 Benzene and Toluene Exposure Concentrations

High exposure concentrations were chosen based on the current rodent LOAEL data for both benzene (10 ppm) and toluene (80 ppm) to test whether the effects would differ in a bird as compared to a rodent. The low exposure concentrations were based on data from air monitoring near flare stacks in southern Alberta (Carmalt 2005). The average concentrations of benzene (0.0845 ppm) and toluene (0.0852 ppm) measured over spring and summer months were considered. Then the concentration representing the environmental exposure was increased by 10 times the ratio of benzene: toluene (1:8) was kept consistent, and final low-dose concentrations were benzene 0.1 ppm and toluene 0.8 ppm.

Praxair Distribution produced benzene and toluene gas mixtures under order number 637748-00 (Geismer, Los Angeles, CA, USA). High-pressure aluminum cylinders with a 4.12m^3 capacity were gravimetrically filled with a breathing-grade air carrier gas and a 10 ppm benzene, 80 ppm toluene mixture (Al BZ10MT1C-AS) (Years 1 and 2) or a 0.1 ppm benzene, 0.8 ppm toluene mixture (Al BZ0.1MT1C-AS) (Year 2). Mixtures were verified to +/- 5% before distribution using gas chromatography with flame ionization detection (GC-FID). Ultra-high pressure steel cylinders with an internal volume of 6.43 m^3 containing breathing grade air (AI BR-K) were also purchased from Praxair (Saskatoon, SK (2005) St Laurent, PQ (2006), Canada) for control group exposures.

3.2.1.4 Daily Standard Operating Exposure Procedure and Total Dose Time

The same standard operating procedure (SOP) was used in dose years 1 and 2 with the exception of the low-dose group addition in year 2.

Between 0800 and 0830, Monday through Friday, control group birds were captured and transported to the inhalation chamber described in Chapter 2.0. Individuals were placed tail-first into the animal holding chambers and the lid was firmly secured. Birds were randomly distributed in the chambers daily. Once the control or dose group kestrels were in place, the breathing grade air cylinder, vacuum pump, breathing grade air inlet gas line ball valve and gas regulator were all turned on. The inlet flow meter was adjusted to 5 LPM. In year 1 the dosing time was 90 min (weeks 1, 2) and 60 min (weeks 3, 4, 5). In both years chamber equilibration and clearing took 7 minutes. Once the dosing session was complete, the birds were returned to their holding rooms with fresh water baths and food. Clearing the chamber of benzene and toluene required just breathing grade air to be run for 7 min before turning off the system and removing the birds. The next dose group was then placed into the chambers and the same steps were followed. In 2006 the control group (breathing grade air) was followed by the low dose and then finally the high dose groups. In 2005 the high dose group followed the control group. The dosing time in year 2 was 90 min with 7 min each for equilibration and detoxification.

In 2005 the test animals were exposed for 1-1½ hr per day for 28 days for a total exposure time of 34 h and in 2006 the exposure was 1½ hr per day for 27 days totaling 40.5 h.

3.2.2 Humoral Immune Response

All birds used as test subjects in 2006 had been previously vaccinated with dinitrophenol-keyhole limpet hemocyanin (DNP-KLH), (Calbiochem, Terochem Laboratories Ltd, Edmonton, AB) DNP-KLH as juveniles in a 2005 study to determine immune effects associated with in ovo exposure to PBDEs. The first vaccination in 2006 would therefore elicit a secondary (IgG) rather than primary (IgM) immune response.

3.2.1.1 Immunization of American kestrels with Dinitrophenol-Keyhole Limpet Hemocyanin

Sensitization was accomplished using a DNP-KLH vaccine formulated per bird as follows: 6.7µl of stock DNP-KLH in MOPS (7.5 mg/ml in 3-[N-morpholino] propane sulfonic acid (MOPS)) added to 30.8 µl sterile phosphate buffered saline (PBS) plus 37.5 µl of Emulsigen-D adjuvant (Emulsigen with dimethhyldioctadecyl ammonium (DDA) bromide) (private source). In a polypropylene tube the DNP-KLH was added to the PBS and then the Emulsigen-D was mixed in. The entire solution was emulsified on ice using an 18 gauge needle attached to a syringe under a laminar flow hood.

Immunizations were administered as follows: A baseline plasma sample (pre-sensitization) was taken before each bird received 2 subcutaneous (50µl total - between shoulder blades, inner thigh region) and 1 intramuscular injections (25 µl in breast muscle) of the DNP-KLH vaccine. One week (7 days) after the initial vaccination a blood sample was drawn to measure the primary IgM response and a second booster injection was administered as described. Seven days following the booster a blood sample was collected to quantify the secondary antibody response.

Weeks 4 (pre-) and 5 (primary) blood samples were collected using the protocol stated in section 3.2.1.2 using jugular venipuncture. The last sample (secondary) was

taken via intracardiac puncture while the bird was anesthetized with halothane prior to euthanization as per the authorized animal care protocol.

3.2.1.2 ELISA Detection of Primary and Secondary Antibody Responses

To quantify the anti-DNP-KLH antibody response an enzyme-linked immunosorbent assay (ELISA) was performed follows.

A total of 100 µl of 0.5 µg/ml DNP-KLH in carbonate buffer (0.05 M, pH 9.5) was added to all wells of a 96-well Nunc-Immuno Maxisorp microtiter plate (Canadian Life Technologies, Inc., Burlington, ON, Canada) and incubated at 4 C for 15-16 h. After rinsing the plate 4 times with 0.05 M PBST (pH 7.2: 0.05% Tween 20) and tapping dry, residual binding sites were blocked using 100 µl of bovine serum albumen (BSA) (0.25% BSA in 0.05 M PBS, pH 7.2: 0.05% Tween 20 (PBS-T). This was incubated for 30 min at 38 C. The plates were then washed as previously described. All samples were run in duplicate. Plasma from 5 kestrels was diluted in 100 µl PBS, and then serial double dilutions were made from 1:50 to 1:6400 across the plates. These were incubated for 60 min at 38 C. This preliminary run was used to create a standard from kestrel sera that had moderate to high levels of anti-DNP-KLH antibody levels. This pooled serum was then run on every plate as a control or standard curve for comparison with the unknown samples. After washing the plate, 100 µl of a 1:400 dilution of laboratory-raised rabbit anti-kestrel (Smits and Baos 2005) antibody in PBS-T was added to the plate and incubated for 30 min at 38 C. After washing the plates, 100 µl of a 1:800 dilution, goat anti-rabbit, peroxidase conjugated, secondary antibody (Sigma Aldrich, St. Louis, MO, USA) in PBS-T was added to all wells and incubated for 30 min at 38 C. Finally the plates were washed and 100 µl of 2, 2'-azino-bis (3-ethylbanzo-thiazoline-6-sulfonic

acid) (ABTS) (Mandel Scientific, Guelph, ON, Canada) was added to all wells and incubated in the dark for 5 min. Using 1% sodium dodecyl sulfate (SDS) in ultrapure water, the reaction was stopped and plates were read on a BioRad 3550 microplate reader with a 405 nm filter (BioRad Laboratories, Missassauga, ON, Canada) to determine the optical density (OD_{405}) of each sample. Data were analyzed using microplate manager version 4.0 (BioRad Laboratories, Missassauga, ON, Canada).

Prior to running samples from all test birds a linear range was determined for a subset of individuals from the control, low, and high-test groups. The mean absorbance value (OD_{405}) of each sample was extrapolated from the plot derived from the set of reference standard concentrations vs. the reference standard OD values as determined by a four-parameter quadratic curve fit. Correlation coefficients (R^2 values) below 0.95 were not accepted. The resulting value was the primary or secondary antiDNP-KLH antibody level in individual kestrel plasma.

3.2.3 Cell-Mediated Immunity Using the Delayed Type Hypersensitivity Test

Years 1 and 2

3.2.2.1 Pre-sensitization to Dinitrophenol-Keyhole Limpet Hemocyanin

To elicit a proper delayed-type hypersensitivity (DTH) response that is a true measure of integrated cell-mediated immunity the animal must have prior sensitization to the test compound. The prior exposure to DNP-KLH provided this sensitization and a DTH response could be elicited anytime greater than two weeks post-vaccination. One week prior to DTH challenge all birds had a 1-cm patch on the mid-patagium (wing web) of the left wing cleared of all feathers and down.

3.2.2.2 Skin challenge with Keyhole Limpet Hemocyanin

Prior to dosing on the last day of exposures, birds were weighed and the patagium (plucked one week previously) was measured (mean of three measurements mm) using a micrometer (Dyer OD gage 0·01 mm, The Dyer Company, Lancaster, PA, USA), swabbed with alcohol, and injected with 20µg/50µl PBS of keyhole limpet hemocyanin (mcKLH) (Pierce Biotechnology Inc., Rockford, IL) using a 30 gauge needle. Injection sites were marked with a waterproof marker.

3.2.2.3 Measurement of the Cell Mediated Response

Twenty-four hours later, birds were weighed and injection sites were measured by the same investigator. The DTH response was the difference between the post and pre skin thickness.

3.2.4 Granuloid Cell Populations in Bone Marrow

Year 2

On the 27th study day 8 birds from each group were euthanized for further studies. The tibiotarsus from each bird was removed and an impression smear was prepared from the bone marrow onto glass slides. Six impressions were made for each sample. Slides were fixed with a May-Grunwald stain (Clinical Pathology Lab, Hematology Dept, WCVM, U of S, Saskatoon, SK). Smears from all exposure groups from 2006 were evaluated in a blind study by a board-certified clinical pathologist at the WCVM (Dr. Moira Kerr, Clinical Pathologist, Prairie Diagnostic Services, Saskatoon, SK, Canada).

Two 500-cell differential counts were performed on the impression smears. The granuloid (primarily heterophil precursor) cell populations were evaluated based on mitotic and post-mitotic activity pools. The mitotic pool included myeloblasts,

promyelocytes, and myelocytes. The post-mitotic pool included metamyelocytes, band heterophils and mature heterophils. These counts describe a maturation index or the ratio of proliferating to mature granulocytes.

3.2.5 Peripheral Blood Differential Leukocyte Counts

Year 2

Blood smears were routinely prepared on bevel-edged glass slides (VWR International, Mississauga, ON, Canada) for all birds. The slides were then air-dried, stained with Wright-Giemsa and cover-slipped (Clinical Pathology Lab, Hematology Dept, WCVM, U of S, Saskatoon, SK, Canada).

Differential white blood cell (WBC) counts were based on 100 cell counts in five separate fields under 1000X oil magnification using a Clay Adams laboratory counter. The percentage of heterophils, basophils, eosinophils, lymphocytes, and monocytes were estimated as was the total white blood cell concentration/mm^3 (Equation 3-1)(Campbell 1995a;Thrall 2004). The individual leukocyte percentages and total white blood cell concentration (10^9 per L) were then used to calculate the total individual leukocyte concentrations (10^9 per Litre). The packed cell volumes (PCV) for all test birds were within the normal range of 35%-55% so the following formula was used to calculate the total WBC concentration.

Estimated WBC/mm^3 = (Average # of WBC/5 fields * 3,500,000[a])/1000[b] **(3-1)**

[a]Approximate number of erythrocytes per mm^3 in birds with normal PCVs
[b]Average number of erythrocytes in five monolayers 1000X fields

3.2.6 Thyroid Hormones Levels

Years 1 and 2

Competitive enzyme immunoassay (EIA) kits designed for thyroid hormone quantification in humans were purchased from Cedarline Laboratories. Triiodothyronine (T3) and Thyroxine (T4) levels in plasma were determined using separate kits with T3 (07BC-1005, MP Biomedicals, Diagnostics Division, Orangeburg, NY, USA) or T4 (07BC-1007 MP Biomedicals, Diagnostics Division, Orangeburg, NY, USA) specific enzyme conjugates. The assay procedure and reagent preparation were nearly identical and are described as follows.

As a pilot study, five individuals' serum samples were run in duplicate to determine if the plasma concentration of thyroid hormones in birds was in the linear range of the reference standard. The concentrations for the T3 standard curve were between 0ng/mL and 10ng/mL. Thyroxine plasma concentrations are generally higher not lower so the standard curve values ranged from 0μg/dL and 25μg/dL. All kestrel values were within these ranges (data not shown) so the kits were used according to the Cedarlane Laboratories standard operating procedure (SOP).

With all reagents and assay materials at room temperature, 50μl (T3) or 25μl (T4) of serum samples and standards were added in duplicate to the T3 or T4 secondary antibody (goat anti-mouse IgG) coated microtiter wells. To this 50 μl of antibody reagent was added and mixed for 30 sec (T3 assay only), then 100 μl of the previously prepared working conjugate was added, mixed for 30 sec, and incubated at room temperature for 60 min. Then all wells were washed 5 X with Millipore water and tapped dry. One hundred μl of 3, 3'5, 5'-tetramethylbenzidine (TMB) was added to each well, incubated

in the dark for 20 min at room temperature and subsequently stopped by adding 100µl of the provided solution. After mixing for 30 sec the absorbance was read at 450 nm on a Spectramax 190 microplate reader (Molecular devices Corporation, Sunnyvale, CA, USA) using Softmax Pro version 4.0 software for analysis. The mean absorbance value (OD_{450}) of each sample was extrapolated from the plot derived from the reference standard concentrations vs. the reference standard OD values. The resulting value was the concentration of T3 (ng/ml) or T4 (µg/dL) in individual kestrel plasma.

3.2.7 Vitamin A Levels

Dose Years 1 and 2

3.2.7.1 Plasma Retinol

Plasma samples collected from birds before and after dosing with benzene and toluene during the summers of 2005 and 2006 were stored at -20 degrees C in amber-coloured, eppendorf microcentrifuge tubes (VWR International Ltd. Mississauga, ON, Canada) until analysis. Due to the photo-oxidation properties of retinoids, all samples were analyzed in an enclosed, dark work space with a 20 watt gold fluorescent light bulb (Bulbtronics Inc. Farmingdale NY). All solvents, unless otherwise stated, were HPLC Grade (99%) and purchased from VWR International or EMD Chemicals.

All samples were extracted and analyzed in the following manner. Samples were thawed in the dark and microcentrifuged (Brinkman instruments Eppendorf Microcentrifuge, 5415D, VWR International, Mississauga, ON) for 5 min X 2300 RCF to remove all fibrous debris. Seventy-five µL of plasma was added to a 10 ml screw top Oak Ridge Teflon centrifuge tube (VWR International, Mississauga, ON). To the plasma 50 µl of an 8 µg/ml Retinol acetate (RA) internal standard (R4632 Sigma Aldrich, Oakville

ON, USA) was added for a final RA concentration of 320 μg/L per sample and shaken by hand. Next, 250 ul of 100% ethanol containing 15 mg/ml 2, 6-di-tert-butyl-4-methylphenol (BHT) (Sigma Aldrich, Oakville ON, USA) was added and vortexed for 20 sec. Lastly, 1.5 ml of hexane was added, the mixture was vortexed for 2 min, and then centrifuged at 1258 RCF at 4 degrees C for 2 min (Brinkman Instruments Eppendorf multipurpose Centrifuge, 5810, VWR International, Mississauga, ON) until the organic phase had separated. After centrifugation the upper hexane phase with the extracted retinoids was carefully removed with glass pipettes and transferred to aluminum foil-covered 5 ml glass test tube. The addition of 0.5 ml hexane, centrifugation, and organic layer transfer was repeated 2 more times. Next, the collected hexane layers were removed and evaporated under nitrogen gas for approximately 30 min (Praxair, Saskatoon, SK, Canada). The residual pellet was then dissolved in 500 μl of methanol and ultrasonicated for 5 min in a water bath. The sample containing all lipid soluble vitamins was collected into a 1 ml syringe and filtered using a 0.2 μm PTFE acrodisc premium syringe filter (PALL Life Sciences, VWR International, Mississauga, ON) into a 1.8 ml amber-coloured screw thread glass HPLC vial with 9 mm step vial closures (VWR International, Mississauga, ON).

The vial containing the extracted retinoids was then analyzed by high performance liquid chromatography (HPLC) as follows. The chromatography apparatus consisted of a C18 column (Microsorb-MV, 150 mm X 4.6 mm I.D. Varian, Mississauga, ON) with a 100 % methanol mobile phase at a flow rate of 1 ml per min. Eluants were monitored by ultraviolet absorption using a diode array wavelength detection (PDA model 330 Varian Inc, Mississauga, ON) and fluorescence detection (Fluorometer model

363, Varian Inc, Mississauga, ON. Fifty µl of sample was injected by an autosampler (model 410 Varian Inc, Mississauga, ON) with an automatic post-injection wash phase (60 methanol: 40 water) after each sample, and reached the column via a tri-valve pump (model 230 Varian Inc, Mississauga, ON) with a dynamic mixing stage. The wavelength for ultraviolet (UV) absorption was 325 nm and fluorescence was 325 nm for excitation and 480 nm for emission. Total run time was 7 min with a peak retention time for retinol at ~ 3 min and retinyl acetate at ~ 4 min.

Daily calibration curves were prepared using a series of dilutions of retinol (Sigma Aldrich, Oakville ON) (Table 3.1) dissolved in mobile phase with the same concentration of internal standard (retinol acetate 800 µg/L) as samples. Data were analyzed using Galaxy software version 1.8. Actual concentrations of retinol (µg/L) were calculated using linear regression of curve data using measured retinol to internal standard peak area ratios. Only calibration curves with a correlation coefficient (R^2) greater than 0.95 were accepted.

Table 3-1 Standard curve concentrations and reagents used for plasma retinol quantification (µg/L) in benzene- and toluene-dosed American kestrels.

Standard	Methanol (µl)	Retinol acetate (µl)	Retinol acetate [µg/L]	Retinol (µl)	Retinol [µg/L]
1	1800	200	800	0	0
2	1750	200	800	20	10
3	1700	200	800	100	50
4	1600	200	800	200	100
5	1400	200	800	400	200
6	1200	200	800	600	300
7	1000	200	800	800	400
Blank	2000	0	0	0	0

Table 3-2 Standard curve concentrations and reagents used for liver retinyl palmitate quantification (μg/L) in benzene- and toluene-dosed American kestrels.

Standard	Methanol: Dichloromethane (μl)	Retinol acetate (μl)	Retinol acetate [μg/L]	Retinyl palmitate (μl)	Retinyl palmitate [μg/L]
1	1993.6	6.4	320	0	0
2	1943.6	6.4	320	50	250
3	1893.6	6.4	320	100	500
4	1843.6	6.4	320	150	750
5	1793.6	6.4	320	200	1000
6	1593.6	6.4	320	400	2000
7	1393.6	6.4	320	600	3000
8	1193.6	6.4	320	800	4000
Blank	0	0	0	0	0

Table 3-3 Gradient method used for the elution of retinol acetate (internal standard) and retinyl palmitate for liver retinyl palmitate quantification (μg/L) in benzene- and toluene-dosed American kestrels.

Time (min)	DCM %	MeOH %	Stage
0	0	100	pre-run
1	0	100	run
5	0	100	run
6	0-30	70-100	gradient equilibration
12	30	70	run
13	0-30	70-100	gradient equilibration
14	0	100	post-run equilibration

3.2.7.2 Liver Retinyl Palmitate

Liver samples collected in the summers of 2005 and 2006 were frozen in liquid nitrogen and stored in a – 80 degree C freezer in clear screw-cap microtubes (VWR International, Mississauga, ON, Canada) until extraction and analysis based on a revised protocol (Milne and Botnen 1986) in the fall. Due to the photo-oxidation properties of retinoids, all samples were handled as described in Section 3.2.7.1.

Liver samples were thawed and weighed before extraction. Approximately 0.7 mg of liver was ground in a large mortar and divided into 2 equal portions (0.2 mg homogenized liver) that would serve as duplicates. Samples were then transferred (independently) to a small mortar and ground with anhydrous sodium sulfate (FW 142.02 g /mole, Sigma Aldrich, Oakville ON) until a homogeneous dry pink powder was observed. The liver sodium sulfate mixture was then transferred to a Teflon tube (VWR International, Mississauga, ON) and 800 μl of a 10 μg/ml retinyl acetate internal standard (320 μg/L final concentration) (R4632 Sigma Aldrich, Oakville, ON, Canada) was added. Next, 7 ml of a 9:1 methanol: dichloromethane (MeOH: DCM) mixed organic extraction solvent was added and the solution was vortexed (5 min) and ultrasonicated in a water bath (5 min). The mixture was then centrifuged at 1258 RCF at 4 C for 2 min (Brinkman Instruments Eppendorf multipurpose Centrifuge, 5810, VWR International, Mississauga, ON, Canada) and the organic phase was poured into an aluminum-covered volumetric flask (VWR International, Mississauga, ON). The extraction phase was repeated 2 more times, each involving the addition of 7 ml of MeOH: DCM, centrifugation and removal of the organic phase. After all the organic layers containing the retinyl palmitate had been collected a volumetric flask was filled to a 25 ml final volume with MeOH: DCM. The

sample was drawn into a 1 ml syringe, filtered with a 0.2 μm PTFE acrodisc premium syringe filter (PALL Life Sciences, VWR International, Mississauga, ON) and expelled into a 1.8 ml amber-coloured screw thread glass HPLC vial with 9 mm step vial closures (VWR International, Mississauga, ON).

The vial containing the extracted retinyl palmitate was then analyzed by HPLC as described above with the following differences. The chromatography apparatus consisted of a C18 column (Microsorb-MV, 150 mm X 4.6 mm I.D. Varian, Mississauga, ON) with a 100 % methanol to 30% dichloromethane: 70% methanol gradient mobile phase at a flow rate of 1 ml – 1.5 ml per min (Table 3.3). Eluants were monitored by ultraviolet absorption using a diode array wavelength detection (PDA model 330 Varian Inc, Mississauga, ON) and fluorescence detection (Fluorometer model 363, Varian Inc, Mississauga, ON. Fifty μl of sample was injected by an autosampler (model 410 Varian Inc, Mississauga, ON) with a post-injection wash phase (60 methanol: 40 water) after each sample, and reached the column via a tri-valve pump (model 230 Varian Inc, Mississauga, ON) with a dynamic mixing stage. The wavelength for ultraviolet (UV) absorption was at 336 nm and fluorescence was 336 nm for excitation and 480 nm for emission. Total run time was 13 min with a peak retention time for retinol acetate at ~ 4 min and retinyl palmitate at ~ 10 min.

A calibration curve was prepared daily using a series of dilutions of retinyl palmitate (Sigma Aldrich, Oakville ON) (Table 3.2) dissolved in mobile phase with the same concentration of internal standard (retinol acetate 320 μg/L) as samples. Data were analyzed using Galaxy software version 1.8.Actual concentrations of retinol palmitate on a wet weight basis (μg/L) were calculated using linear regression of curve data using

measured retinol to internal standard peak area ratios. Only calibration curves with a correlation coefficient (R^2) greater than 0.95 were accepted.

3.2.8 Statistical Analysis

Results were analyzed using SPSS (version 15.0, SPSS Inc., Chicago, IL, USA). The unit of analysis was the control, or benzene and toluene exposure groups. Data were initially tested for normal distribution using non-parametric Kolmogorov-Shmirnov (ks) analysis and homoscedasticity (homogeneity of variance) using Levene's test. Any p value from the ks or Levene's tests with a value < 0.05 was log transformed and re-analyzed using a one-way ANOVA. If transformation was not possible the data were analyzed using a Kruskal-Wallis and/or Chi squared non-parametric test with a Mann-Whitney U post-hoc analysis model to compare groups.

For normally distributed data a descriptive summary was generated and outliers (greater than mean +/- 2 SD) were eliminated (Boxenbaum.HG *et al.* 1974). A one-way ANOVA was initially run on all data sets to determine any group effects followed by a general linear model (GLM) with a univariate analysis (ANCOVA). Before ANCOVA analysis a GLM tested covariates for significant interactions with the variable of interest. A line graph depicting the variable vs. covariate interaction with markers set at treatment groups was also used to visualize suitable covariates ($p > 0.05$). Once covariates were validated, an ANCOVA with all valid confounders was run and non-significant covariates were iteratively removed until only significant covariates remained. Bonferroni or Fisher's Least Significant Different (LSD) post-hoc analysis adjusted for experiment-wise error was used to ensure the Type I error rate associated with multiple

outcomes was no greater than α 0.05. Significance was $p < 0.05$ with a trend at $p < 0.1$ (Fernie *et al.* 2005b).

Year 2

In addition to the benzene and toluene dose-dependent effects, PBDE exposure through the parents had to be considered in analyses. A 2-way ANOVA was used to test the effects between benzene/toluene treatment and PBDE treatment in relation to the variable of interest. Any significant value considered due to PBDE exposure could not be considered a valid measure of effect due to benzene and toluene exposure.

3.3 Results

3.3.1 Antibody Mediated Immunity

Years 1 and 2

There were no differences between benzene/toluene-dosed birds and control kestrels in 2005 for primary ($F_{1,19} = 0.000$, $p = 0.995$) or secondary ($F_{1,19} = 0.600$, $p = 0.449$) anti-DNP-KLH antibody levels (Figure 3-1).

The 2006 studies on previously immunized kestrels showed very different results. Antibody responses were different after the first immunization among benzene/toluene-dosed groups ($F_{2,27} = 3.363$, $p = 0.051$). Both the low-dose ($p = 0.041$) and high-dose ($p = 0.031$) groups had higher antibody response than control group birds (Figure 3-2 a, b). After the booster a decrease in anti-DNP-KLH antibodies was seen in the high-dose group ($p = 0.004$) (Figure 3-2 d) but not the low-dose group ($p = 0.193$) (Figure 3-2 c).

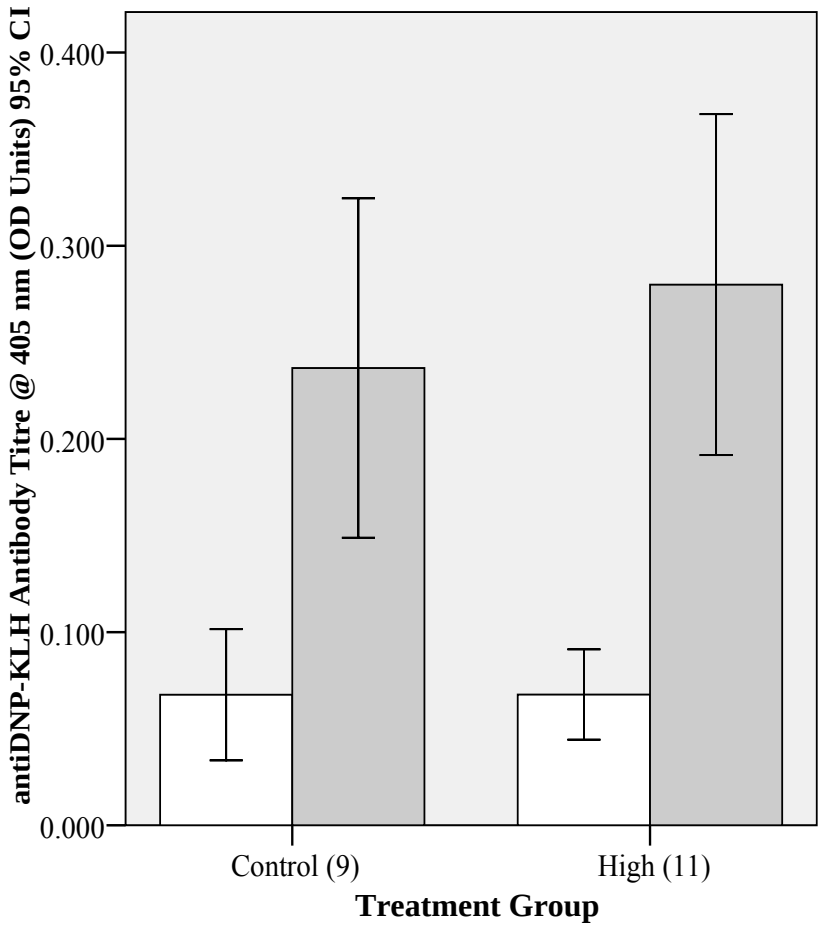

Figure 3-1 Anti- Dinitrophenol-Keyhole Limpet Hemocyanin primary (light bars) (p = 0.995) and secondary (dark bars) (p = 0.449) antibody levels (OD Units) in American kestrels exposed to breathing grade air (control) and high-dose (benzene-10 ppm and toluene-80 ppm) in 2005. Vertical bars represent the mean anti-DNP-KLH antibody value for each dose group. The number of individuals per exposure group is presented in parentheses.

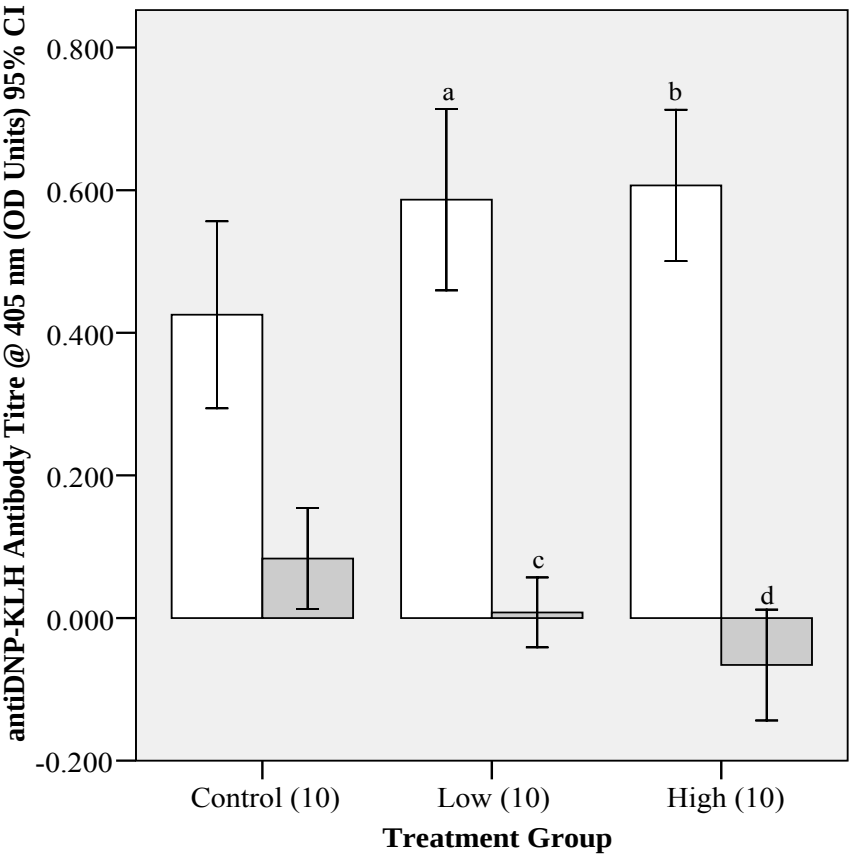

Figure 3-2 Anti-DNP-KLH secondary (light bars) (p = 0.051) and continued secondary (dark bars) (p = 0.005) antibody levels (OD Units) in previously immunized American kestrels exposed to breathing grade air (control), low-dose (benzene-0.1 ppm, toluene-0.8 ppm), and high-dose (benzene-10 ppm and toluene-80 ppm) in 2006. Vertical bars represent the mean antiDNP-KLH antibody value for each dose group. Significant differences compared to controls are represented by a (p = 0.041), b (p = 0.031), d (p = 0.004). The number of individuals per exposure group is presented in parentheses.

3.3.2 Cell-Mediated Immunity (Delayed Type Hypersensitivity Test)

Years 1 and 2

Delayed type hypersensitivity (DTH) responses (mm) to KLH were significantly suppressed in benzene/toluene-dosed groups in 2005 ($F_{1,23} = 5.604$, $p = 0.028$) (Table 3-3, Figure 3-3 a) and 2006 ($F_{2, 31} = 9.647$, $p = 0.001$). Both exposure years, exposed birds had a suppressed cell-mediated immune response ($F_{1,23} = 5.604$, $p = 0.028$) (Table 3-3, Figure 3-3 a). In 2006 the findings were repeated, with a dose related suppression of the DTH response ($F_{2, 31} = 9.647$, $p = 0.001$). In 2006, the high-dose group ($p = 0.0004$) (Figure 3-4 b) had a poorer DTH response than the low-dose group ($p = 0.028$) (Figure 3-4 a), both of which were lower than control birds. None of the tested covariates (post injection weight, SSI, TSI, T3, and T4 levels) significantly interacted with the DTH response.

3.3.3 Bone Marrow Granuloid Lineage Cytology

Year 2

Birds exposed to high-dose benzene and toluene showed a trend towards increased granulocytic mitotic index (GMI) in the bone marrow compared with both control and low-dose birds although this was not strong statistically ($F_{2, 21} = 2.690$, $p = 0.095$) (Figure 3-5). Fisher's LSD revealed as significant increase ($p = 0.038$) when compared to low-dose individuals and a trend compared to control birds ($p = 0.101$).

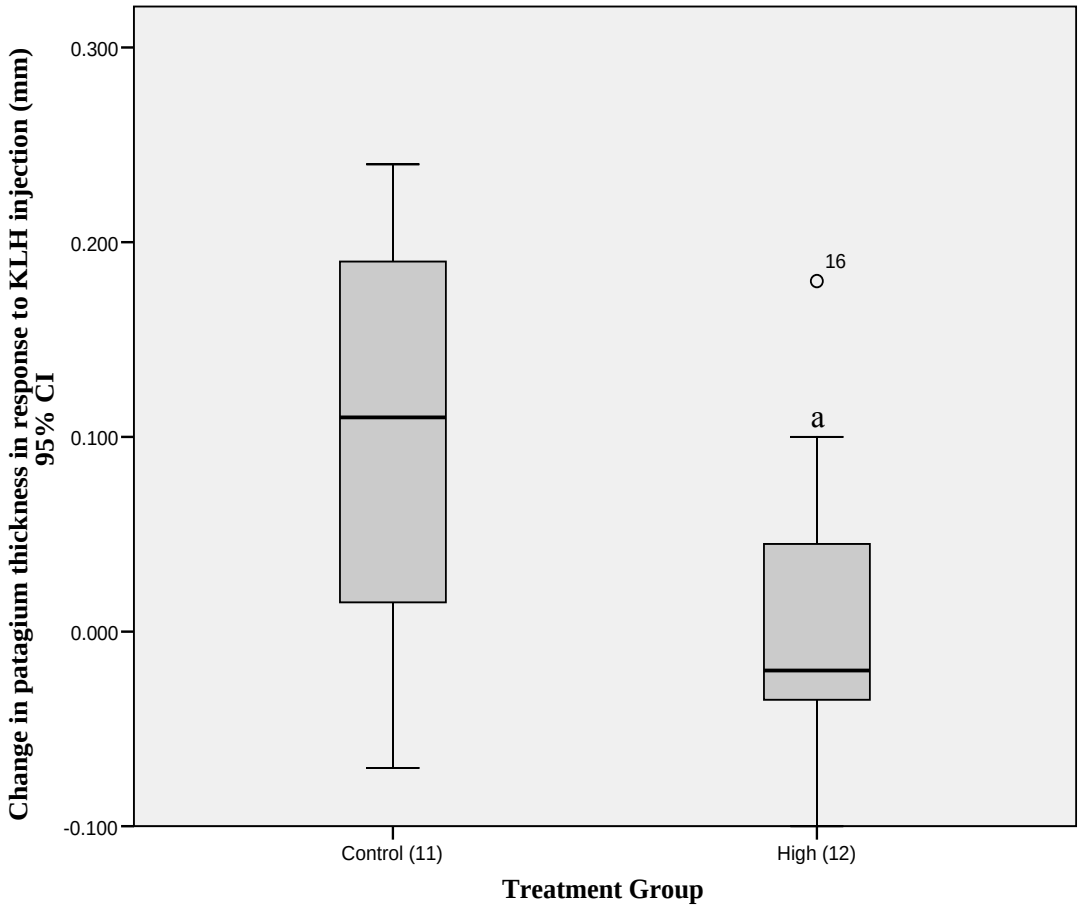

Figure 3-3 The delayed type hypersensitivity response (mm) in American kestrels exposed to breathing grade air (control) and high-dose (benzene-10 ppm and toluene-80 ppm) in 2005. Open circles depict outliers. Superscript a is significantly lower (p = 0.028). The number of individuals per exposure group is presented in parentheses.

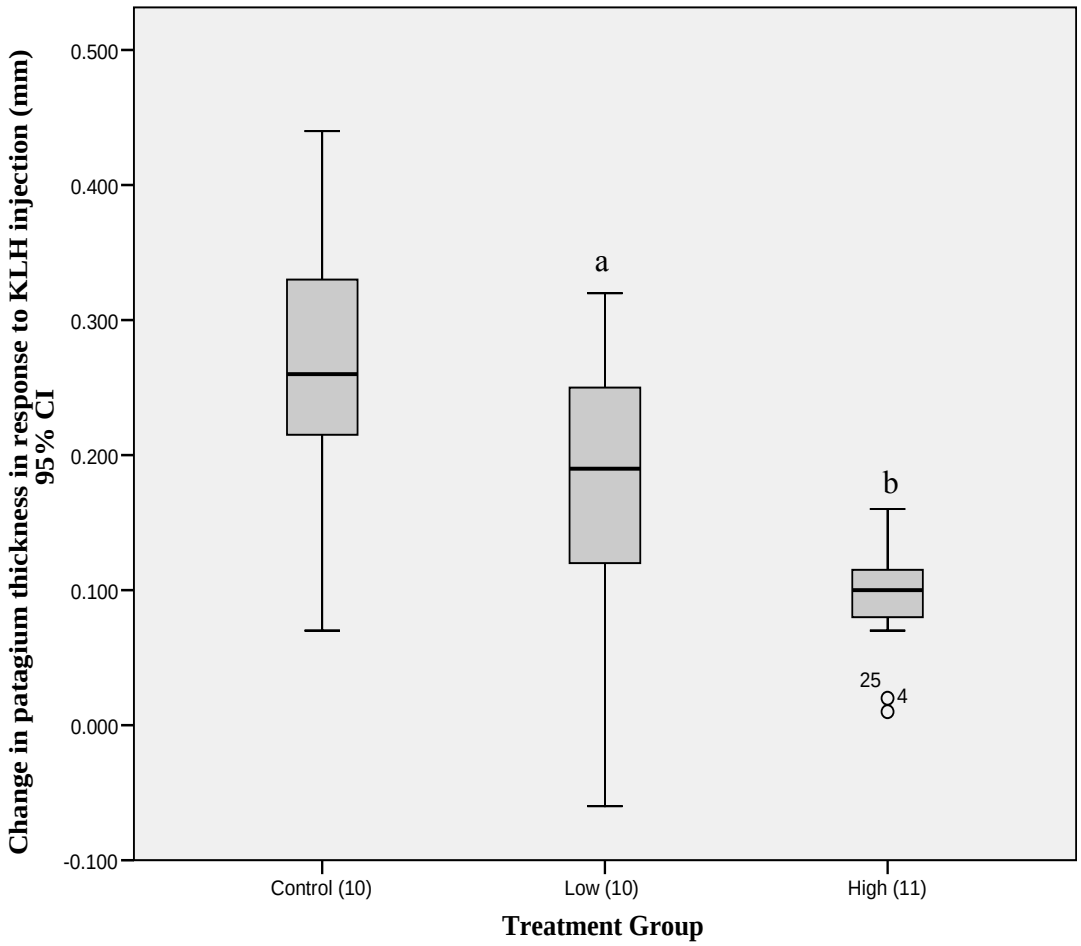

Figure 3-4 The delayed type hypersensitivity response (mm) in American kestrels exposed to breathing grade air (control), low-dose (benzene-0.1 ppm, toluene-0.8 ppm), and high-dose (benzene-10 ppm and toluene-80 ppm) ($p = 0.001$) in 2006. Open circles depict outliers. Low dose suppression is shown by a ($p = 0.028$) and high dose by b ($p = 0.004$). The number of individuals per exposure group is presented in parentheses.

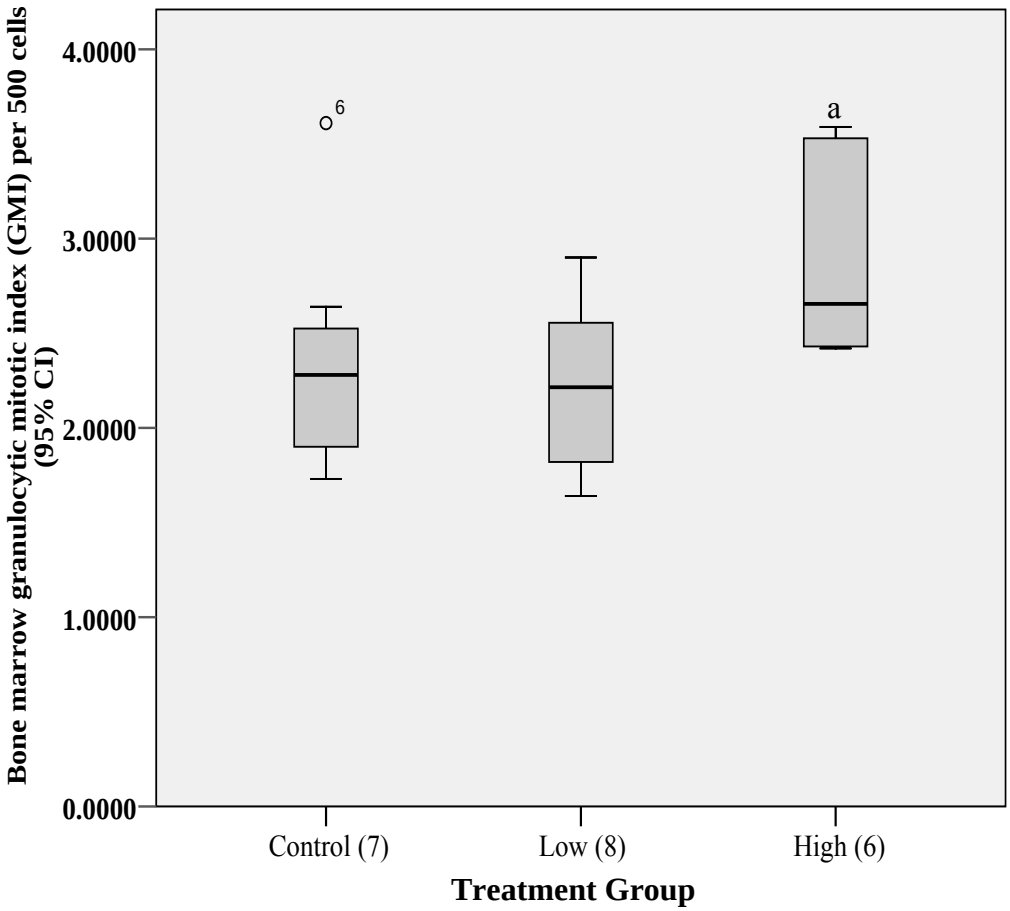

Figure 3-5 Bone marrow granulocytic maturation index (GMI) in American kestrels exposed to to breathing grade air (control), low-dose (benzene-0.1 ppm, toluene-0.8 ppm), and high-dose (benzene-10 ppm and toluene-80 ppm) ($p = 0.095$) in 2006. Horizontal bars represent the mean change in the granulocytic maturation index (ratio of mitotic to post mitotic cells) for each dose group. Open circles depict outliers. The increase in high-dose individuals maturation index is represented by a ($p = 0.038$ compared to low dose, 0.101 compared to control individuals). The number of individuals per exposure group is presented in parentheses.

3.3.4 Peripheral Blood Leukocyte Differentials

Year 2

ANCOVA analysis of high- and low-dose birds as compared to control birds showed significant increases in both eosinophil ($F_{2,22} = 4.126$, $p = 0.032$) and basophil ($F_{2,22} = 5.954$, $p = 0.009$) cell populations. Bonferroni post hoc analysis shows that low-dose individuals had a higher percentage of eosinophils ($p = 0.008$) (Figure 3-7 a) and basophils ($p = 0.030$) (Figure 3-7 b). A trend towards increased monocytes was also noted ($F_{2,22} = 2.682$, $p = 0.093$) (Figure 3-8 a). Table 3-4 presents the leukocyte descriptive statistics and species comparisons.

Table 3-4. Descriptive statistics for white blood cell populations in the circulation of American kestrels exposed to benzene and toluene and the available reference ranges for the same cell populations in similar species.

	Mean	SD	Lower 95% CI	Higher 95% CI	F	df	Significance
American kestrel							
Eosinophils	0.11	0.091	0.068	0.147	3.609	22	0.046
Basophils	0.1	0.07	0.072	0.133	5.475	22	0.013
Monocytes	0.25	0.17	0.174	0.321	2.682	22	0.093
White blood cell count	3	1.42	2.369	3.596	0.743	22	0.488
Red-tailed hawk							
Eosinophils	1.963	1.51	0.051	9.612	N/A	N/A	N/A
Basophils	0.567	0.463	0.034	2.352	N/A	N/A	N/A
Monocytes	0.911	0.772	0.031	3.315	N/A	N/A	N/A
White blood cell count	12.95	5.853	3.1	29.1	N/A	N/A	N/A
Peregrine falcon							
Eosinophils	0.2	0.106	0.058	0.468	N/A	N/A	N/A
Basophils	0.343	0.323	0.045	1.14	N/A	N/A	N/A
Monocytes	0.606	0.58	0.063	2.941	N/A	N/A	N/A
White blood cell count	10.02	4.792	2.25	23.5	N/A	N/A	N/A

All cell types are presented as total concentrations (10^9 per L)

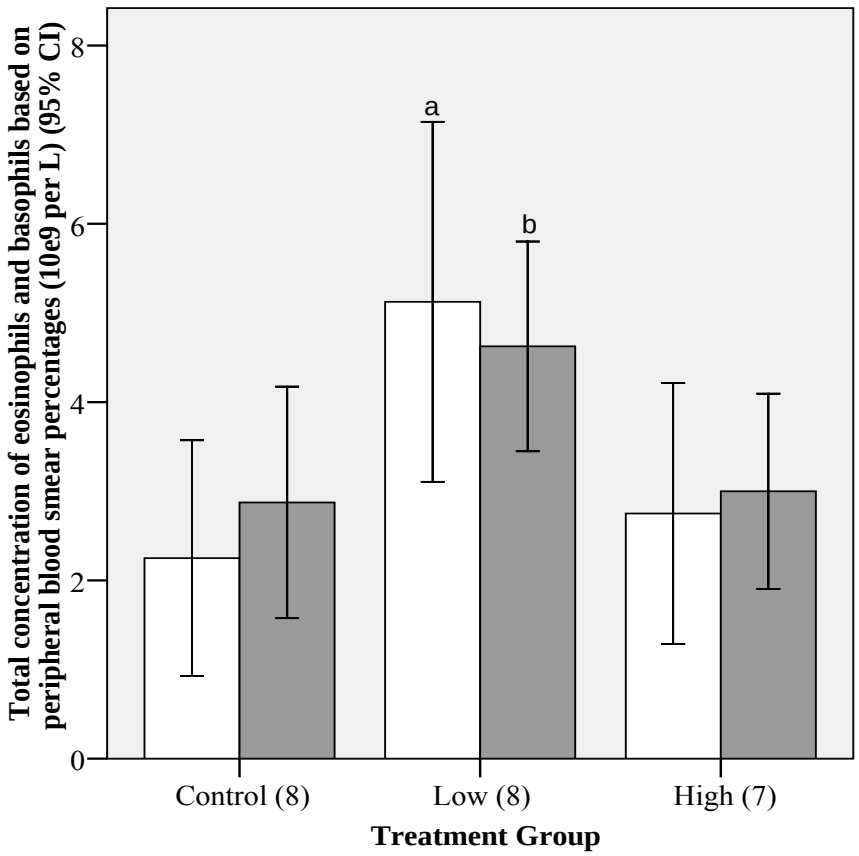

Figure 3-6 Eosinophil (light bars) and basophil (dark bars) concentrations (10^9/L) in American kestrels exposed to breathing grade air (control), low-dose (benzene-0.1 ppm, toluene-0.8 ppm), and high-dose (benzene-10 ppm and toluene-80 ppm) in 2006. Vertical bars represent the mean concentrations for each dose group. The increase in the percent of eosinophils in the low-dose group as compared to control group individuals is shown by a ($p = 0.03$) and basophils by b ($p = 0.008$). The number of individuals per exposure group is presented in parentheses.

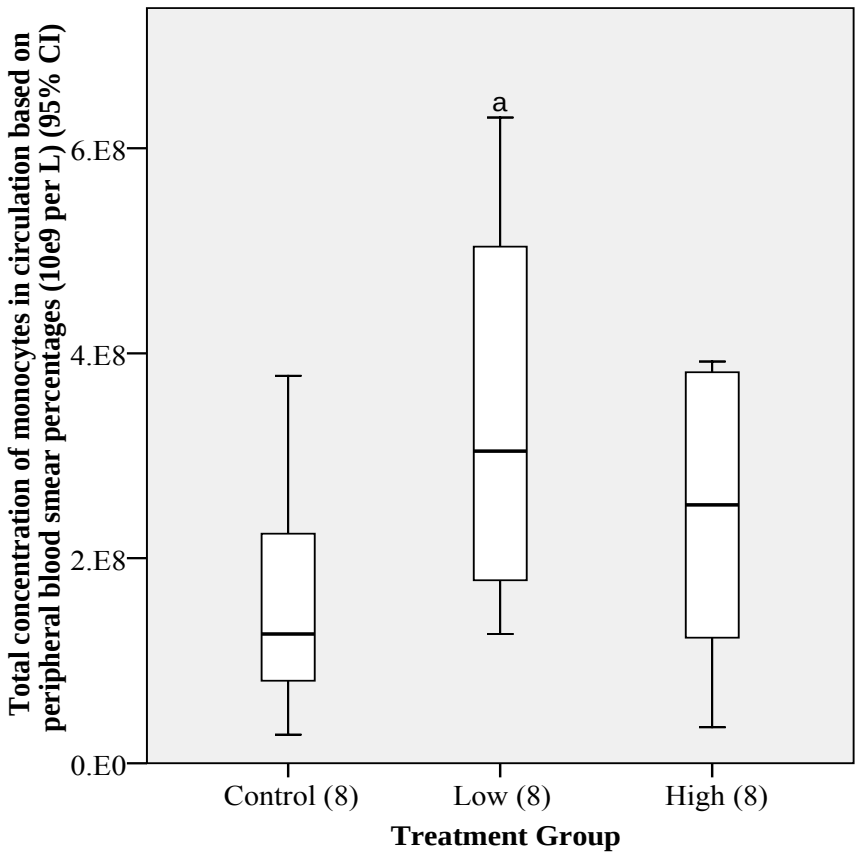

Figure 3-7 Monocyte concentrations (10^9 per L) in American kestrels exposed to breathing grade air (control), low-dose (benzene-0.1 ppm, toluene-0.8 ppm), and high-dose (benzene-10 ppm and toluene-80 ppm) in 2006. Vertical bars represent the mean concentrations of either eosinophils or basophils for each dose group. The increase in the percent of monocytes is shown by a ($p = 0.095$). The number of individuals per exposure group is presented in parentheses.

3.3.5 Thyroid Hormones

Years 1 and 2

In 2005, the levels of triiodothyronine (T3) (μg/L) after exposure to benzene and toluene show a decreasing trend in high-dose birds ($F_{1, 21} = 3.450$, $p = 0.079$) when the pre-dosing T3 levels were used as a covariate of analysis. These results did not repeat in 2006 (Table 3-5). Unfortunately in 2006, after the birds had been randomly assigned to dose groups, the pre-dosing levels were found to be significantly different between the control and exposed groups ($F_{2.31} = 6.674$, $p = 0.004$). This effect was not PBDE-dependent.

3.3.6 Vitamin A

Serum Retinol Levels

In 2005 there was significant decrease in the retinol levels (μg/L) in high-dose birds after benzene and toluene exposure ($t(10) = 3.282$, $p = 0.008$) (Figure 3-9 a). The same was seen in 2006 individuals exposed to high doses of benzene (10 ppm) and toluene (80 ppm) (($t(7) = 2.389$, $p = 0.048$) (Figure 3-10 a). The paired samples analyses are shown in Table 3-5. Plasma retinol levels in high-dose birds after dosing were lower than the same individual's retinol levels before dosing.

Liver Retinyl palmitate levels

There were no differences in liver retinyl palmitate levels with respect to dose group in either dose year (Table 3-6).

Immune Organ Somatic Indices

Years 1 and 2

There were no differences between thyroid, spleen, or adrenal (2005 only) somatic indices between dose groups (Table 3-6).

Table 3-5 Paired T-test analysis of pre-dosing vs. post-dosing plasma retinol levels (μg/L) in benzene- and toluene-dosed birds in 2005 and 2006.

	Mean Difference	SD	Lower 95% CI	Higher 95% CI	t	df	Significance[a]
2005							
Control	13.271	46.741	-18.130	44.672	0.942	10	0.369
High[b]	49.419	49.938	15.870	82.968	3.282	10	0.008
2006							
Control	-0.187	66.975	-83.347	82.973	0.006	4	0.995
Low[c]	3.853	76.629	-60.210	67.916	0.142	7	0.891
High	37.954	44.939	0.384	75.523	2.389	7	0.048

[a] 2-tailed significance
[b] 10 ppm benzene, 80 ppm toluene
[c] 0.1 ppm benzene, 0.8 ppm toluene

Table 3-6. Descriptive statistics for thyroid hormone levels, retinol palmitate levels, and organ somatic indices in American kestrels exposed to benzene and toluene.

	Mean	SD	Lower 95% CI	Higher 95% CI	F	df	Significance[a]
2005							
Thyroxine (T4) (ug/ml)	7.052	6.96	3.97	10.14	2.019	21	0.737
Triiodothyronine (T3) (ng/ml)	1.562	0.371	1.4	1.73	0.116	21	0.171
Retinol palmitate (ug/ml)	2729.94	1591.02	1811.311	3648.56	0.797	13	0.390
Liver somatic index (g)	0.0226	0.0026	0.0214	0.0238	0.026	20	0.873
lung somatic index (g)	0.0094	0.0009	0.009	0.0098	0.731	20	0.403
kidney somatic index (g)	0.008	0.0008	0.0076	0.0083	1.149	20	0.297
thyroid somatic index (g)	0.00012	0.00004	0.00009	0.00014	2.025	19	0.172
spleen somatic index (g)	0.0008	0.0004	0.0006	0.001	0.051	20	0.824
brain somatic index (g)	0.0234	0.0016	0.0227	0.0242	0.864	20	0.364
testis somatic index (g)	0.00007	0.00002	0.00005	0.00008	0.384	14	0.546
2006							
Thyroxine (T4) (ug/ml) pre-dosing	1.819	0.177	1.755	1.882	6.674	31	0.004
Thyroxine (T4) (ug/ml) post-dosing	1.912	0.206	1.837	1.986	0.179	31	0.837
Triiodothyronine (T3) (ng/ml) pre-dosing	0.778	0.506	0.596	0.961	1.82	31	0.180
Triiodothyronine (T3) (ng/ml) post-dosing	1.611	0.456	1.446	1.775	0.12	31	0.887
Retinol palmitate (ug/ml)	5583.47	5921.58	2957.99	8208.96	0.082	21	0.922
Liver somatic index (g)	2.052	0.245	1.947	2.159	0.131	22	0.878
lung somatic index (g)	0.765	0.103	0.72	0.809	2.071	22	0.152
kidney somatic index (g)	0.664	0.083	0.628	0.699	0.073	22	0.93
thyroid somatic index (g)	0.011	0.009	0.007	0.015	0.411	22	0.668
spleen somatic index (g)	0.035	0.011	0.0299	0.039	1.152	22	0.336
brain somatic index (g)	2.485	0.125	2.431	2.539	1.22	22	0.316
testis somatic index (g)	0.016	0.01	0.012	0.021	0.975	22	0.394

[a] One way Anova

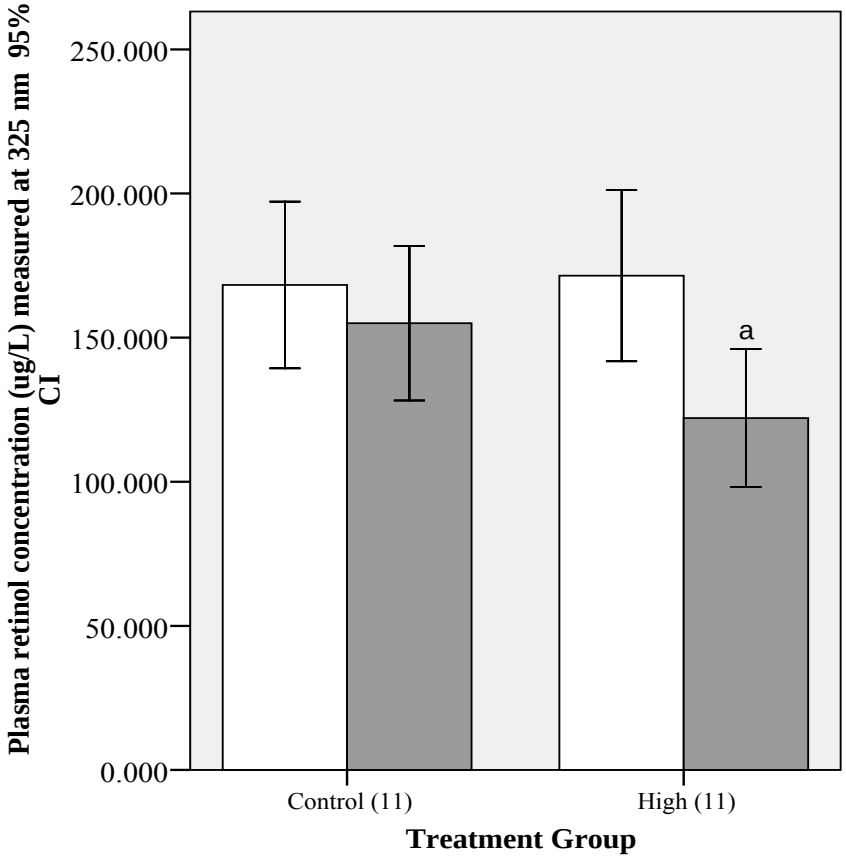

Figure 3-8 Change in plasma retinol concentrations (μg/L) in American kestrels exposed to breathing grade air (control) and high-dose (benzene-10 ppm and toluene-80 ppm) in 2005. Pre-dosing retinol levels (light bars) and post-dosing retinol levels (dark bars). Vertical bars represent the mean retinol concentration for each dose group. The decrease in plasma retinol in the high-exposure group is represented by a ($p = 0.008$). The number of individuals per exposure group is presented in parentheses.

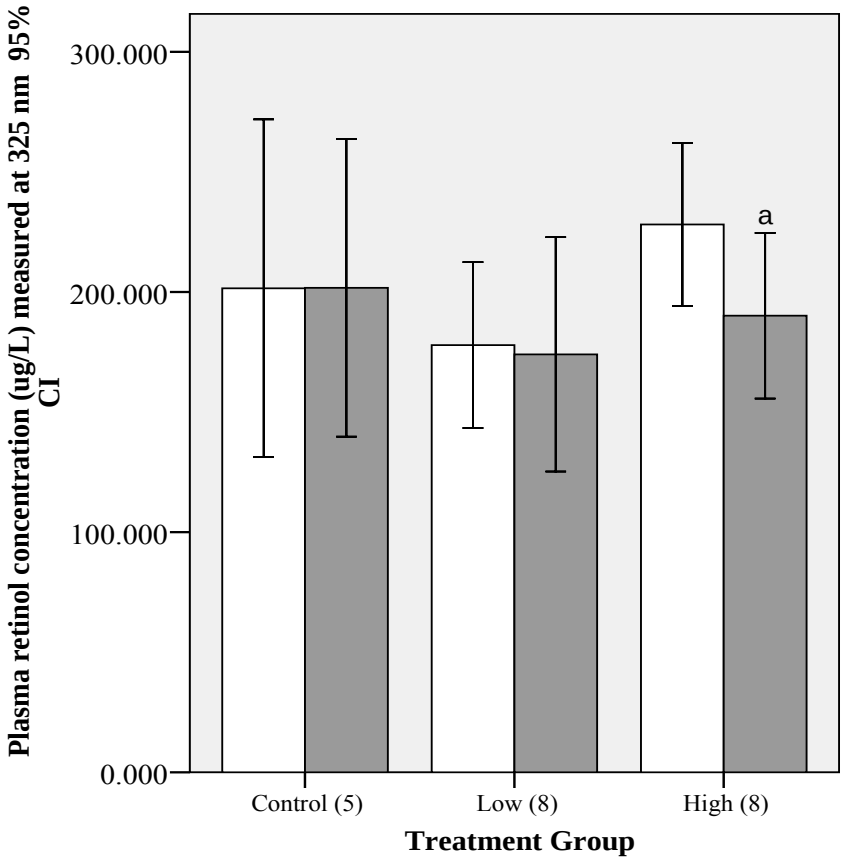

Figure 3-9 Change in plasma retinol concentrations (μg/L) in American kestrels exposed to breathing grade air (control), low-dose (benzene-0.1 ppm, toluene-0.8 ppm), and high-dose (benzene-10 ppm and toluene-80 ppm) in 2006. Pre-dosing retinol levels (light bars) and post-dosing retinol levels (dark bars).Vertical bars represent the mean retinol concentration for each dose group. The decrease in plasma retinol in the high-exposure group is represented by a ($p = 0.048$). The number of individuals per exposure group is presented in parentheses.

3.4 Discussion

Antibody and Cell Mediated Immunity

In the current study there was no effect on humoral immunity in wild birds exposed to 10 ppm benzene and 80 ppm toluene in 2005**.** However, in previously immunized captive birds exposed to the same concentrations there was a significant increase in the secondary antiDNP-KLH response and a decrease in the maintenance of antiDNP-KLH antibody levels after the booster vaccination. A study of humans exposed to 3-7 ppm benzene showed a similar pattern with an increased primary antibody responses (Lange *et al.* 1973). The decrease in secondary levels may represent a suppression of immunoglobulin producing cells by benzene but is more likely due to the over stimulation of the humoral immune system by repeat vaccinations. The birds in all treatment groups in 2006 had been previously immunized as chicks to study the effects of PBDEs in 2005. The differences between year 1 and 2 could also be attributed to the wild vs. captive status of the test birds or the difference in total dose time between 2005 (34 h) and 2006 (40.5 h). The results show no effect in year 1 but increased humoral response in year 2. This may also be explained in part by the decreased cell-mediated response.

The cell-mediated immune response as measured by an antigen-dependent delayed-type hypersensitivity response is a better reflection of the integrated immune system *in vivo* (Tizard 1992) **.** The CMI measured here is a response by DNP-KLH sensitized individuals with memory T lymphocytes, which are able to respond to a KLH antigenic stimulation. Macrophages and dendritic cells are responsible for antigen processing and presentation and the subsequent inflammatory reaction caused by infiltration of the memory T lymphocytes. The cell mediated reaction is T lymphocyte

CD4 (Th1) sub-population mediated whereas antibody reactions are generally T lymphocyte CD8 (Th2) mediated (Tizard 1992). In 2005 the high-dose birds had a significantly decreased CMI response, as did both the low- and high-dose birds in 2006. This suppression was expected since CD4 T helper lymphocytes have been shown to be more sensitive to the toxic effects of benzene than CD8 T suppressor lymphocytes in rats (Robinson et al. 1997). The results of a DTH test are difficult to interpret as only one previous study has presented the usefulness of the DTH test in birds (Fairbrother and Fowles 1990). Historical data conclusively shows benzene to be a suppressor of the CMI (Farris *et al.* 1997; Ross 2000; Sul *et al.* 2002; Qu *et al.* 2002) so the results presented here are consistent with mammalian studies.

When one arm of the immune system is suppressed another may overcompensate and produce an increased response. A study by (Smits et al. 1996) described the same immunomodulatory phenomenon in mink exposed to pulp mill effluent. She proposes that exposure to an immunotoxicant may enhance one arm of the immune response, while another is suppressed, a theory which is also described by (Bretscher 1981). It is possible that exposure to benzene and toluene in some way promotes a humoral response and suppresses the cell mediated response.

Lymphocytes are sensitive to the toxic effects of benzene's phenolic metabolites and have recently been used as one of the more sensitive indices with which to assess benzene-associated toxicity (Robinson *et al.* 1997; Farris *et al.* 1997). By measuring the circulating immunoglobulin levels and the delayed-type hypersensitivity reactions of animals *in vivo*, the current study has used functional tests of immunocompetence rather than only structural components to understand the effects of benzene on the acquired

immune systems of kestrels. These data are more reliable as they represent actual function of the defense system and as such provides insight into the capability of these birds to deal with foreign antigens and possible infections.

Associations between contaminant exposures and suppressed immune function leading to increased mortality or decreased reproductive success have been demonstrated in many wildlife species. As examples, Glaucous gulls (*Larus hyperboreus*) exposed to organochlorines (OCs) had a higher incidence of intestinal nematode infections ((Sagerup *et al.* 2000), elevated PCB concentrations were positively correlated to the death of striped dolphins (*Stenella coeruleoalba*) in the Mediterranean sea during a morbillivirus outbreak (Aguilar and Raga 1993; Aguilar and Borrell 1994), and altered immune functions in Caspian terns (*Hydroprogne caspia*) exposed to OCs led to deformities, embryonic lethality, and decreased recruitment into the breeding population (Mora *et al.* 1993; Grasman *et al.* 1996a).

Altered immune function and mortality or morbidity events in the wild are difficult to link to laboratory-generated data. Smits and Bortolotti (2001) have found decreased mitogenic PHA-induced T lymphocyte immunity and increased DNP-KLH antibody titers in laboratory studies of American kestrels exposed to PCBs. These findings are supported by a field study that showed the same immunomodulation, a suppressed PHA response and enhanced SRBC antibody response in Caspian terns (Grasman and Fox 2001). These studies show that laboratory- and field-generated data correlate with respect to altered immune function and both are often required to explain as well as predict toxicity.

The current study used wild and captive American kestrels in a laboratory setting to show that low or high benzene and toluene exposure can lead to suppressed T lymphocyte and increased B lymphocyte immune responses which could possibly affect the ability of a wild bird population ability to deal with epizootic or stressful events that could occur in nature.

Bone Marrow Granuloid Cell Population

Bone marrow histopathology is one of the most frequently used methods to establish benzene-related immunotoxicity (Irons *et al.* 1979b; Tunek *et al.* 1981; Tunek *et al.* 1982; Rozen and Snyder 1985; Plappert *et al.* 1994; Abraham 1996; Bernauer *et al.* 2000; Yoon *et al.* 2001). The changes can be in relation to the progenitors of the granulocytic, white blood cell, or red blood cell lines and may be increased or decreased depending on the species, strain, exposure duration, and exposure concentration (ATSDR 2006). It is important to remember these cells can inhabit three separate compartments, the blood, the bone marrow, and various tissues.

The current study found a slightly increased granulocytic maturation index (GMI) in high dose group individuals ($p = 0.095$), which must be interpreted with caution, as the significant increase in ratio is only apparent when compared to low-dose birds. The maturation index for low-dose and control birds was approximately 2 post-mitotic cells: 1 mitotic cell and the high-dose birds had 3 post-mitotic cells per 1 mitotic cell**.** In birds exposed to high doses of benzene/toluene there is an increase in the number of post-mitotic granulocytic cells. In mammals, the maturation index of individuals with synchronous maturation is 1:4 (Thrall 2004). There are no data available for the normal GMI in American kestrels, so the current study must use the control group as its standard.

It is impossible to discern if the expansion of the post-mitotic pool of granulocytes in the high dose group is happening at the time of sampling or was due to an earlier event. Once cells are committed they must complete the maturation cycle (Stockham and Scott 2002). There is no evidence of increased granulocyte numbers in the high dose group individual's circulation. Once cells are released to the blood they may be signaled to immediately enter tissue, which would give the appearance of "normal" circulating numbers. It is also possible that the cells had not yet been released from the marrow hence the increased GMI and "normal" circulating granulocyte populations. The timing of sampling of the bone marrow is integral in determining the overall dysfunction (Jacobs and Valli 1988; Harvey 2001; Stockham and Scott 2002). There was no difference in total WBC numbers between dose groups implying the signal for expansion was before the sampling period. This could also explain why there was no difference in the GMI in low dose birds.

Bone marrow cellularity and maturation profiles are difficult to interpret in relation to benzene toxicity. Various studies in humans, rats, and mice have reported a multitude of contradictory effects. An increased GMI was seen in mice exposed to 10 ppm benzene (Green *et al.* 1981; Farris *et al.* 1997), contradictory to this a decreased GMI was noted in mice exposed to 10-13 ppm benzene (Toft *et al.* 1982). Aksoy, 1972 reported both hypoplasia and hyperplasia in 52 leukemia patients caused by benzene exposures (Aksoy *et al.* 1972a; Aksoy *et al.* 1972b) and hypercellularity of the bone marrow from low-level short-term exposure has also been reported (Snyder *et al.* 1981; Rozen and Snyder 1985).

Peripheral Leukocyte Differential Counts

The basophilic ($p = 0.008$) and eosinophilic ($p = 0.030$) leukocyte responses were significantly increased in low-dose group birds and a trend towards increased monocytes was also present ($p = 0.095$). In order to properly interpret the individual leukocyte percentages they must be analyzed relative to the total WBC concentrations and compared to the pre-established range of the particular cell type for like species (Table 3-6). As the WBC concentration increases or decreases, the individual leukocyte cell concentrations should follow the same pattern. Eosinophils, basophils, and monocytes were all increased in the circulation of low-dose birds but there was no evidence of a shift in the bone marrow towards the granulocytic line. When individual cell concentrations for American kestrels were compared to basophil, eosinophil, WBC, and monocyte levels in Red-tailed hawks (*Buteo jamaicensis*) and Peregrine falcons (*Falco peregrinus)* they were not within the reference ranges (International Species Information System, ISIS, 2002) (Table 3-4). Therefore the control birds were used as the standard for comparison.

There was no evidence to suggest the GMI was increased in low-dose birds but more importantly the actual cell types within the granulocyte lineage were not quantified. It is difficult to draw conclusions, as we do not know if there was or wasn't a shift towards eosinophil, basophil, and monocyte production and decreased heterophil production in the bone marrow. The lack of an increase in the granulocyte: erythrocyte ratio as would also be expected when these numbers are seen in circulation may also be explained by the above statement but it is more likely that the eosinophils, basophils, and

monocytes are not leaving the vascular compartment due to interference with signaling from the tissue compartment.

There are few causes of eosinophilia in birds. Intramuscular injections of antigen- have been linked to eosinophilic reactons while intravenous administration resulted in anaphylaxis (Maxwell 1985). The current study used an intramuscular administration route that could have increased the number of circulating eosinophils but no data were collected on injection sites. Eosinophils are also involved in the early inflammatory DTH reaction, but the low-dose group had a significantly decreased response as compared to controls, thus not explaining the eosinophilia.

Basophils are important in systemic anaphylaxis and cutaneous hypersensitivity reactions, neither of which was present in any individuals in the current study. Climate changes and food restriction can cause basophilia in poultry, but the relationship is not known in kestrels and neither was a factor in the current study (Maxwell and Robertson 1995). It has also been shown that basophilia may develop during periods of high thermal exposures (Maxwell 1993; Maxwell and Robertson 1995) . The increased temperatures experienced within the chamber were consistent between exposure groups and would not explain the increase in the low-dose group basophil numbers.

Vitamin A Status

In both years of the current study there was a notable decrease in the circulating levels of retinol but no apparent effect on retinyl palmitate hepatic stores. Benzene's metabolism and Ah receptor association can possibly explain the alteration. Once benzene enters the body it is metabolized by phase I enzymes in the lung and liver and transported to the bone marrow. In the bone marrow it is biotransformed by

myeloperoxidase to semi-quinone radicals which stimulate the production of reactive oxygen species (Gut *et al.* 1996a). In order to deal with the increased free radical load, plasma retinol and vitamin A precursors would be preferentially relocated to the bone marrow where they inhibit neoplastic transformation by quenching the ROS activity (Sies and Stahl 1995). Oxidative stress was not tested so this remains as speculation in explaining the decreased retinol in high-dose individuals. In all likelihood there may have been toxicity that did not deplete hepatic stores due to compensation by a high Vitamin A diet. Cockerels fed to the kestrels are, a source very rich in vitamin A (Rolland 2000).

The reductions in retinoid levels in plasma in high exposure birds could be responsible for the immunomodulation present. Low vitamin A has been shown to cause reduced antibody production and defective T lymphocyte responses (Friedman and Sklan 1997). The CMI was significantly decreased in birds with decreased retinol levels, an important finding considering that an immune system cannot function properly with low levels of retinol. If one interprets the increased GMI with respect to the decreased levels of retinol in high dose individuals it may be that whatever cellular event occurred to cause an increased GMI might have happened due to decreased circulating levels of retinol.

Reductions in Vitamin A analogues have been reported in birds exposed to dioxins and furans (Spear *et al.* 1992), PCBs (Grasman *et al.* 1996b), various aryl hydrocarbon (Ah) inducing chemicals (Bishop et al. 1999), and PBDEs (Fernie et al. 2005b). Mechanistically, this appears to be a displacement of retinol from TTR in conjunction with increased phase II metabolism, resulting in an increased clearance of

retinol, decreased resorption by the kidney, and increased mobilization of hepatic stores all resulting in decreased retinyl palmitate levels in the liver (Fernie et al. 2005b).

Thyroid Hormone Status and Organ Somatic Indices

The decrease in T3 in high dose birds in 2005 may have been a spurious finding, as it was not seen in captive kestrels in 2006. If the decrease had been in T4 levels then it could be postulated that the concurrent decrease in retinol levels were both due to a decrease in TTR binding. Retinol and T4 share the same carrier, TTR, for transportation through the blood (Rolland 2000). This however was not the case. The difference was considered not biologically significant and due to the wild status of the birds. The diffrences in pre-dosing T3 and T4 levels in captive birds may be due to the stress associated with dividing the birds into new groups and imposing a restructuring of the existing hierarchy.

3.5 Conclusion

This study showed that the alterations in avian immune function after exposure to benzene are similar to that of mammals. The effects of toluene in this binary mixture, whether additive, synergistic, or potentiating, are unknown.

With respect to humoral immunity, captive birds were either more sensitive to benzene than wild birds, or the decreased dosing period in 2005 was substantial enough to not directly affect antibody production. Cell mediated immune function however, was clearly a target for benzene toxicity with the most pronounced effects in high-dose individuals. High exposure kestrels had the greatest degree of cell-mediated (2005, 2006) and antibody-mediated (2006) modulation as well as the greatest decrease in plasma retinol levels.

Cells of the granulocytic lineage were affected both systemically and in the bone marrow**.** It is difficult to interpret these results, as the bone marrow effects of benzene are highly variable, there is no standard for granulocyte levels in the systemic circulation for American kestrels.

To conclude, the American kestrel has proven to be a sensitive model for benzene toxicity. The use of wild versus captive birds must be addressed as well as consistent dose times and total exposures must be ensured for proper analysis of data. The results show suppression of various functions of the immune system (cell-mediated, humoral, innate) and one regulator of the immune system (vitamin A status). The adverse effects are indicative of the sensitivity of birds to inhaled toxicants, because of their unique respiratory physiology. If the immunomodulation witnessed in this laboratory study is occurring in a wild setting, the affected birds may not be able to adequately deal with infectious challenges.

CHAPTER 4

CYTOCHROME P450 ACTIVITY, HAEMATOPOIETIC ALTERATIONS, AND BEHAVIOURAL EFFECTS ASSOCIATED WITH THE INHALATION EXPOSURE OF AMERICAN KESTRELS TO BENZENE AND TOLUENE.

4.1 Introduction

Chemically VOCs in flare gas are unsaturated cyclic (aromatic) hydrocarbons that contain one or more benzene rings (ATSDR 2005). Benzene, toluene, ethylbenzene, and xylene (BTEX) are often used as representatives of this group. Benzene and toluene are hazardous because of their inherent toxicities, wide use in industry, and high volume of production which leads to substantial environmental releases (Robinson *et al.* 1997). They have therefore been selected as the chemicals of concern for this study addressing the toxic effects of airborne contaminants on wild birds.

Raptors have high fidelity to breeding territory (Janes 1984) which on the prairies is often in close proximity to flare stacks. Because of their unique respiratory physiology which results in higher absorption of gases from inspired air relative to mammals (Brown *et al.* 1997) birds in general may be more susceptible to the adverse effects of benzene and toluene in flare gas. This increased susceptibility may lead to greater toxicity than that seen in human (Schechter *et al.* 1989; Schechter *et al.* 1990) and cattle populations (Waldner *et al.* 2001; Waldner 2001).

Section 1.2 outlines the mechanism of action for benzene and toluene, their chemical interactions and the repurcussions of their biotransformation. Sections 1.3 and 1.4 describe the vulnerability of avian species exposed to these chemicals and the reasoning behind the species choice.

Considering the high concentration and immunotoxic potential of benzene and other halogenated hydrocarbons such as toluene in ambient air on the prairie and boreal regions of Saskatchewan and Alberta (Waldner *et al.* 2001) a laboratory based inhalation study was designed to test a sensitive bird species. Birds have a more sensitive respiratory tract than do mammals, which results in relatively more inhaled contaminants being absorbed and metabolized by the lung (Brown *et al.* 1997). The kestrel serves as a useful sentinel species owing to its small size, non-endangered status, easy maintenance in captivity, and extrapolating efficacy to other larger prairie inhabiting falcons (Wiemeyer and Lincer 1987)

The objectives of this study were to determine the effects of benzene and toluene on specific cytochrome P450 monoxygenase enzymes (CYP1A, 2B) that biotransform benzene and toluene in mammalian species, study effects on the haematopoeitic system (in circulation and in the bone marrow) and examine behavioural alterations associated with inhalation exposure to to environmentally relevant concentrations (benzene 0.1 ppm, toluene 0.8 ppm) and rodent LOAELs (benzene 10 ppm, toluene 80 ppm) of benzene and toluene.

4.2 Materials and Methods

Refer to Section 3.2.1.1 for animal care and supply protocol numbers and SOPs.

4.2.1 Inhalation Exposure of American kestrels to Benzene and Toluene

Refer to Chapter 3, Section 3.2.1.

4.2.2 Enzyme Activity

4.2.2.1 Microsome preparations from kestrel liver and lung

Dose Years 1 and 2

Liver and lung tissues from 24 American kestrels previously exposed to breathing grade air or benzene- and toluene-contaminated air were collected within 5 minutes of euthanization, placed in 2.0 ml cryotubes (VWR International, Mississauga, ON, Canada), and frozen in liquid nitrogen (Praxair, Saskatoon, SK or Senneville, PQ, Canada). The samples were stored in a – 80 C freezer until analyzed according to techniques described in (Papp *et al.* 2005a) with the following exceptions.

All samples, buffers, and preparation materials were kept on ice for the duration of the microsome extraction. Tissue samples were placed in a -20 C freezer and removed individually for processing to avoid premature thawing. Samples were held on ice until the tissue had thawed enough to easily slip from the tube. A 0.6 – 0.7 g sample was placed in a 2 ml Safe-Grind Potter-Elvehjem tissue grinder glass (Wheaton Science Products, Millville, NJ, USA). One ml of homogenization buffer (0.15 M KCL: 0.02 M HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ph 7.5) was added to the tube and the sample was hand homogenized using 20 – 30 strokes. The homogenate was transferred into a Beckman thick-walled polycarbonate ultracentrifuge tube (Beckman, VWR International, Mississauga, ON, Canada), any residual was rinsed with 2 ml of

homogenization buffer and an additional 3 ml was added to the ultracentrifuge tube for a final volume of 6.5 ml. Twelve samples at a time were ultra centrifuged at 10,000 g for 20 minutes (4 C) (Beckman L8-55M, Ti50 rotor, Beckman Instruments Inc., Mississauga, ON, Canada). Supernatant was then transferred to a clean ultracentrifuge tube ensuring all fat was removed and each sample was equalized to 12 grams with homogenization buffer. The supernatant was further centrifuged at 100,000 g for 60 minutes (4 C) (Beckman L8-55M, Ti50 rotor). For liver samples the supernatant was removed, the remaining pellet loosened, washed with 1 ml of homogenization buffer**,** equalized to 12 g and ultracentrifuged again at 100,000 g for 60 min (2 C). Lung samples were not as fatty and the microsomal protein recovery was less than liver samples so they were not washed. After centrifugation the supernatants were discarded and the pelleted microsomes were resuspended in 0.6 ml Tris buffer with glycerol (0.05 M tris (2-amino-2-hydroxymethyl-1,3-propanediol); 1 mM EDTA (ethylenediamine tetraacetic acid); 20% (V/V) glycerol; pH 7.4). The microsome samples were then stored in 150 µl aliquots in 0.5 ml cryovials (VWR International, Mississauga, ON, Canada) at – 80 C until protein concentration determination and enzyme activity assays were performed.

4.2.2.2 Protein Determination

DC Protein – Lowry Method

A Bio-Rad DC colourimetric protein assay kit (DC Protein Assay Kit II, Bio-Rad Laboratories Ltd, Mississauga, ON, Canada) was used to determine the protein concentration of duck lung and liver microsome preparations using a BSA standard curve. Using the provided protocol the procedure was as follows. Prior to loading each well 20 µl of Reagent S was added to each ml of reagent A (alkaline copper tartrate

solution). To a 96-well Nunc-Immuno Maxisorp microtiter plate (Canadian Life Technologies, Inc., Burlington, ON, Canada) 5 µl of each BSA standard in 0.05 M PBS (phosphate buffered saline pH 7.2) in a 2-fold dilution series from1.4 mg/ml - 0.01 mg/ml (liver) and 0.14 mg/ml – 0.002 mg/ml (lung) was added to the plate in duplicate for extrapolation of unknown samples microsomal protein. Next 5 µl of a 2-fold serial dilution series of undiluted microsome preparations or samples diluted in PBS (to achieve the desired 0.2 – 0.5 mg/ml protein concentration per well) for liver or lung was added (Table 4.1. Lastly 25 µl of reagent A prepared with reagent S previously and 200 µl of reagent B ((dilute Folin reagent) were added, mixed, and incubated for 15 minutes. Absorbances read at 655 nm on a BioRad 3550 microplate reader (BioRad Laboratories, Missassauga, ON, Canada). Data were analyzed using microplate manager version 4.0 (BioRad Laboratories, Missassauga, ON, Canada).

Fluorescamine Method

Total protein concentrations of microsomes were measured using fluorescamine (F9015, Sigma-Aldrich, Oakville, ON, Canada) simultaneous to ethoxy (EROD), benzyloxy (BROD), methoxy (MROD), and pentoxy (PROD) resorufin-O-dealkylase activity determination (Kennedy and Jones 1994). The validated microsomal standard (51.2, 25.6, 12.8, 6.4, 3.2, 1.6, 0.8, 0.4 mg/ml) curve was used to quantify the amount of protein in each sample using an MFX Microtitre Plate Fluorometer (Dynex Technologies, Chantilly, VA, USA).

Simply 4.2 mg of fluorescamine (MW 278.3, N7505, Sigma-Aldrich, Oakville, ON, Canada) was dissolved in 7 ml of HPLC grade acetonitrile shortly before the enzyme activity reaction was stopped (Section 4.2.1.4). Sixty microlitres of the fluorescamine:

acetonitrile solution (36 μg fluorescamine/well) was added to each well and the fluorescence was read at 390 nm excitation and 460 nm emission (BioRad 3550 microplate reader (BioRad Laboratories, Missassauga, ON, Canada)). The fluorescence units for each sample were then extrapolated from the previously generated four parameter logistic duck liver or lung microsome standard curve and reported as total mg of protein per ml of microsomes.

4.2.2.3 Resorufin Formation Assays

Dose Years 1 and 2

Resorufin standards consisted of 12.5, 25, 50, 100, 200, 400, 800, and 1600 nM resorufin in phosphate buffer. The following resorufin substrates were used to measure the corresponding cytochrome P450 isoform activity; Ethoxy (EROD) - CYP1A1, benzyloxy (BROD) - CYP 2B2, methoxy (MROD) - CYP1A2, and pentoxy (PROD) - CYP1A and CYP2B1 activities. These alkoxy resorufin stock substrate solutions (Sigma Aldrich, Oakville, ON, Canada) were previously prepared in dimethylsulfoxide (DMSO) (Sigma Aldrich, Oakville, ON, Canada) and stored at room temperature. The 140 μl reaction volume contained 0.2 to 0.3 mg/ml microsomal protein, 2 μM of substrate, and 0.5 mM nicotinamide adenine dinucleotide phosphate (MW833.35, N7505, NADPH, Sigma Aldrich, Oakville, ON) in phosphate buffer (pH 7.7). The plates were kept on ice and each well was prepared in triplicate with a specific blank as follows. The volumes of liver and lung microsomes added to each well varied between the tissues due to the differential protein content. Liver activity was determined by adding 2.5 μl of microsomes, 77.5 μl of phosphate buffer (pH 7.7), 30 μl of 0.5 mM NADPH and 30 μl of the 4.7 μM alkoxyresorufin substrate working solution. Lung microsomes had a lower

protein concentration therefore 10 µl of microsomes in 70 µl of phosphate buffer (pH 7.7), 30 µl of 4.7 µM alkoxyresorufin substrate working solution, and 30 µl of 0.5 mM NADPH in buffer were required. EROD, BROD, and MROD reactions in liver samples were all incubated in a 39 C waterbath for 10 minutes. Liver microsomes PROD activity proceeded at a slower rate and required a 70 minute incubation time before maximal enzyme formation was achieved (data not shown). Lung microsomes were incubated for 70 minutes for all alkoxyresorufin assays. The reaction was stopped using 60 µl of acetonitrile with 600 ug/ml fluorescamine for protein determination and read with an excitation wavelength of 530 nm and emission wavelength of 590 nm on an MFX Microtitre Plate Fluorometer (Dynex Technologies, Chantilly, VA, USA). The specific blank had an additional 30 ul of phosphate buffer added to replace the NADPH volume. Kinetic incubation studies were determined using 2.5 µl liver microsomes and 10 µl lung microsomes to determine the time at which the maximal product formation occurs.

Raw data was processed by subtracting the specific blank from each sample and then extrapolating the samples resorufin fluorescence units (RFU) from the previously generated resorufin standard curve to determine the amount of product formed (pmol/ml).

The total EROD, BROD, MROD, PROD activity was calculated as resorufin formed per mg of microsomal protein per minute. The formula is presented in Equation 4-1.

Activity = resorufin product (pmol/ml) / protein (mg/ml) * incubation time **(4-1)**

4.2.3 Haematology

4.2.3.1 Polychromatophil Index

Years 1 and 2

Approximately 1 ml (≤1% body mass) of blood was collected from the jugular vein using 7.5% EDTA (2005) or Heparin (2006) coated syringes and 28 gauge needles. Two blood smears per bird were prepared, air-dried, stained with Wright-Giemsa and cover slipped (Clinical Pathology Lab, Hematology Dept, WCVM, U of S, Saskatoon, SK).

Polychromatophilic erythrocytes (immature red blood cells in circulation) were quantified in ten fields per blood smear under 1000X oil magnification (approximately 200 erythrocytes per field (Thrall 2004). The average number of polychromatophils per field was compared between dose groups. The packed cell volume (PCV) and total protein of each blood sample were also determined (microcapillary centrifuge model MB, International Equipment Company, Needham Heights, Massachusetts, USA and refractometer for total protein).

4.2.3.2 Granuloid Cell Populations in Bone Marrow

Year 2

On the 27th day of the inhalation study, 8 birds from each group were sacrificed for detailed studies. The right tibiotarsus from each bird was removed, cracked open, and 6 impression smears per animal were prepared on glass slides. Slides were fixed with a May-Grunwald stain (Clinical Pathology Lab, Hematology Dept, WCVM, U of S, Saskatoon, SK) to allow evaluation of the erythroid precursor cell line. The control, low, and high dose cases from 2006 were evaluated in a blind study by a board certified

clinical pathologist at the WCVM (Dr. Moira Kerr, Clinical Pathologist, Prairie Diagnostic Services, Saskatoon, SK, Canada).

Two 500-cell differential counts were performed on the impression smears. The erythroid cell populations were evaluated by categorizing the cells as mitotic or post-mitotic. The mitotic pool included rubriblast, prorubricyte, basophilic rubricyte, and polychromatophilic rubricyte. Polychromatophilic erythrocytes in the bone marrow were considered post-mitotic. The activity pools were used to derive a maturation index that explains the ratio of proliferating to mature erythrocytes.

4.2.4 Behavioural Analysis

4.2.4.1 Behaviours

Year 1

In 2005, control and benzene/toluene exposed American kestrels were evaluated for neurological effects using a battery of behavioural tests designed around the introduction of a known predator, the Great horned owl (GHO). After 25 days of inhalation exposure birds was released alone into their normal room and video taped from behind a curtain for 3 minutes (ACU, University of Saskatchewan, Saskatoon, SK) as a conditioning session. The bird was then removed and held in a darkened container until all birds in the treatment group had undergone the same treatment. The following day, a stuffed GHO was placed on a shelf two feet higher than the two rope perches present in each of the holding rooms. Birds were released individually and videotaped as before.

Year 2

In 2006, the behavioural evaluation was markedly different. Birds were exposed to a live GHO in a specific enclosure that was different from their normal flight pen in the ASCC (Macdonald Campus, McGill University, Ste Anne de Bellevue, PQ). The owl was housed in a wire mesh cage and was lower than the two rope perches. The interaction between the individual kestrel and the GHO was video taped from behind a plywood door attached to the corner of the room with one-way glass at the observer's height. On day 26, each kestrel was released into the room as a conditioning phase with no predator present and their behaviours were videotaped. The following day the owl was placed in the middle of the northern wall prior to the kestrel being released into the room. The interaction was again taped for 3 minutes.

4.2.4.2 Evaluation of Behaviours

Years 1 and 2

In the fall of 2005 and 2006, six volunteers from the toxicology graduate and undergraduate program with no previous knowledge of the study design or involvement in any aspect of the exposure scored and quantified the following predetermined behaviours for each bird (Balgooyen 1976); time spent in flight (seconds), time spent vocalizing (seconds), the number of head bobs, and number of tail flicks or bobs. Each volunteer recorded the behaviours during both the 3-minute conditioning and 3-minute predator exposure sessions.

4.2.4 Statistical Analysis

All results were analyzed using SPSS (Version 15.0, SPSS Inc., Chicago, IL, USA). The unit of analysis was treatment group; control and low and high benzene and toluene exposure groups. Data were initially tested for normal distribution using non-parametric Kolmogorov-Shmirnov (ks) analysis and homoscedasticity (homogeneity of variance) using Levene's test. Any p value from the ks or Levene's test with a value < 0.05 was logarithm transformed and analyzed using a one way ANOVA. Where transformation of the data was not possible, a Kruskal-Wallis and/or Chi squared non-parametric test with a Mann-Whitney U post hoc analysis model to compare groups was used.

For all normally distributed data a descriptive summary was generated and any outlying value (greater than mean +/- 2 SD) was eliminated from further analyses. A one way ANOVA was initially run on all data to determine any group effects followed by a general linear model (GLM) with a univariate analysis (ANCOVA). Before ANCOVA analysis a GLM was designed to test the effects between treatment and covariates in relation to the variable of interest. A line graph depicting the variable vs. covariate interaction with markers set at treatment groups was also prepared to visualize suitable covariates ($p > 0.05$). Once covariates were validated an ANCOVA with all valid potential confounders was run and non-significant variables were iteratively removed. Bonferroni or Fisher's Least Significant Different (LSD) post-hoc analysis adjusted for experiment wise error and was used in developing the final model to ensure the Type I errors associated with multiple outcomes was not greater than α. The criterion for statistical significance was $p < 0.05$ and a trend was considered at $p < 0.1$.

Simple linear regression was used to determine the relationship between the mitotic and post-mitotic pools of erythroid cells in the bone marrow as well as the relationship between polychromatophils in circulation and in the bone marrow. The normality of variables was tested using a Q-Q plot. The coefficient of determination, R^2, explained how much of the variance of the dependent variable was explained by the independent variable. A p value < 0.05 indicated a significant linear equation and the best fit line are defined by y = mx (B constant) + b (slope) from the unstandardized coefficients table.

Year 2

In addition to the benzene and toluene birds used as test subjects in year 2 had to be statistically analyzed to see if a previous PBDE exposure was a possibly significant covariate. A GLM with a 2-way ANOVA was used to test the effects between benzene/toluene treatment and PBDE treatment in relation to the variable of interest. Any interaction with $p < 0.05$ was considered due to PBDE exposure and could not be considered a valid measure of effects due to benzene and toluene.

4.3 Results

4.3.1 Pulmonary Cytochrome P450 Activities

Year 2

The MROD activity of lung microsomes in American kestrels did not have equal assumed variances ($p = 0.001$) and log transformation did not equalize the data. A Kruskal-Wallis test showed no difference between control and dosed individuals but indicated a possible trend ($p = 0.145$). A post hoc comparison (Mann-Whitney U test) showed no difference in enzyme activities between the control and low dose benzene (0.1 ppm) and toluene (0.8 ppm) birds ($p = 0.645$) but a trend towards a decrease in CYP1A enzyme activity in birds exposed to high doses of benzene (10 ppm) and toluene (80 ppm) ($p = 0.094$) (Figure 4-1 a).

4.3.2 Hepatic Cytochrome P450 Activities

Years 1 and 2

There was no significant difference in any enzyme activity (EROD, MROD, and BROD) between control, high and low exposure birds (Figure 4-2, 4-3).

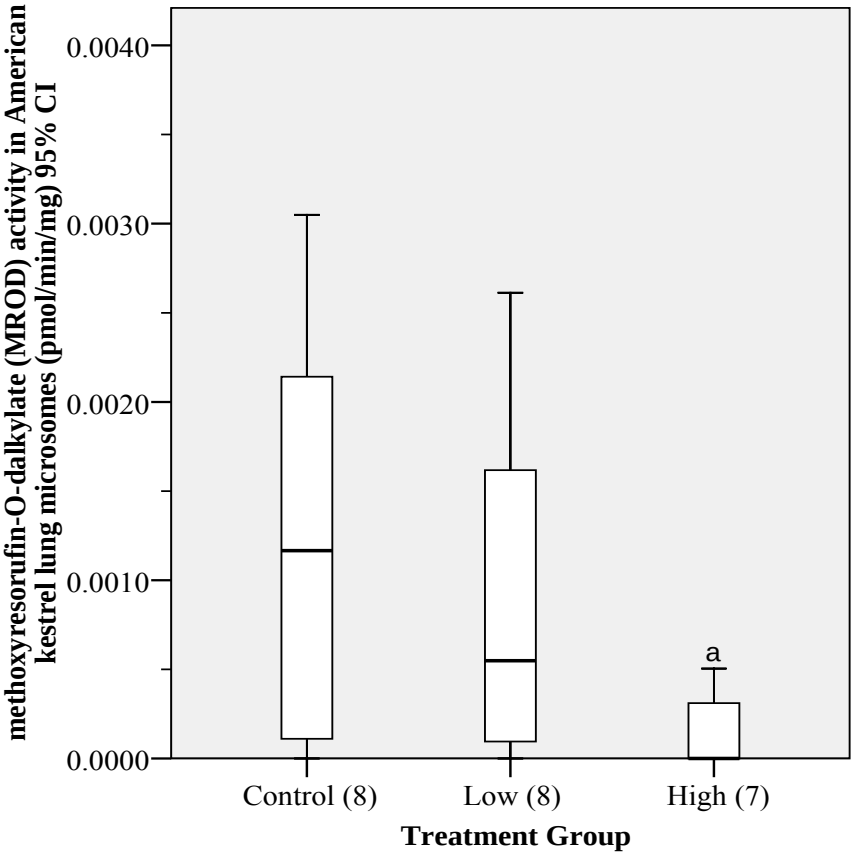

Figure 4-1 Methoxyresorufin-O-dealkylase (MROD) activity measuring CYP1A activity in lung microsomes of American kestrels exposed to breathing grade air (control), low-dose (benzene-0.1 ppm, toluene-0.8 ppm), and high-dose (benzene-10 ppm and toluene-80 ppm) ($p = 0.145$) in 2006. Horizontal bars represent the mean MROD activity. The trend towards decreased CYP1A activity is indicated by a ($p = 0.094$).

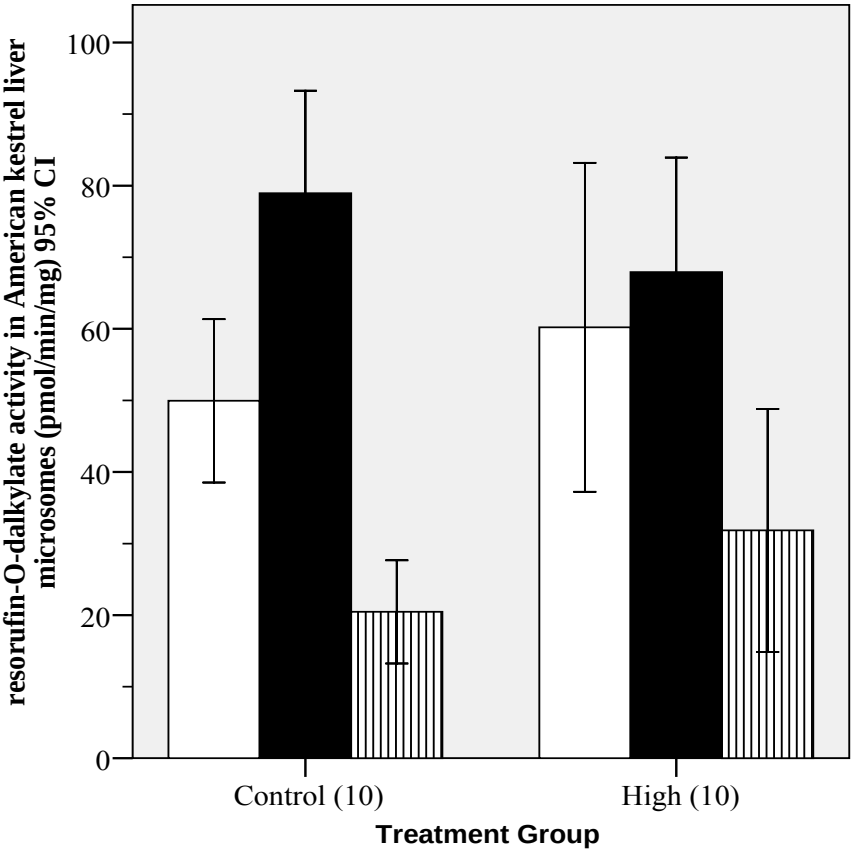

Figure 4-2 Ethoxyresorufin (EROD, CYP1A1) (light bars) (p = 0.398), methoxyresorufin (MROD, CYP1A) (dark bars) (p = 0.268), and benzyloxyresorufin (BROD, CYP2B) (hatched bars) (p = 0.200) O-dealkylase activities in liver microsomes of American kestrels exposed to breathing grade air (control) and high-dose (benzene-10 ppm and toluene-80 ppm) in 2005. Horizontal bars represent the mean O-dealkylase activity. The number of individuals per exposure group is presented in parenthesis.

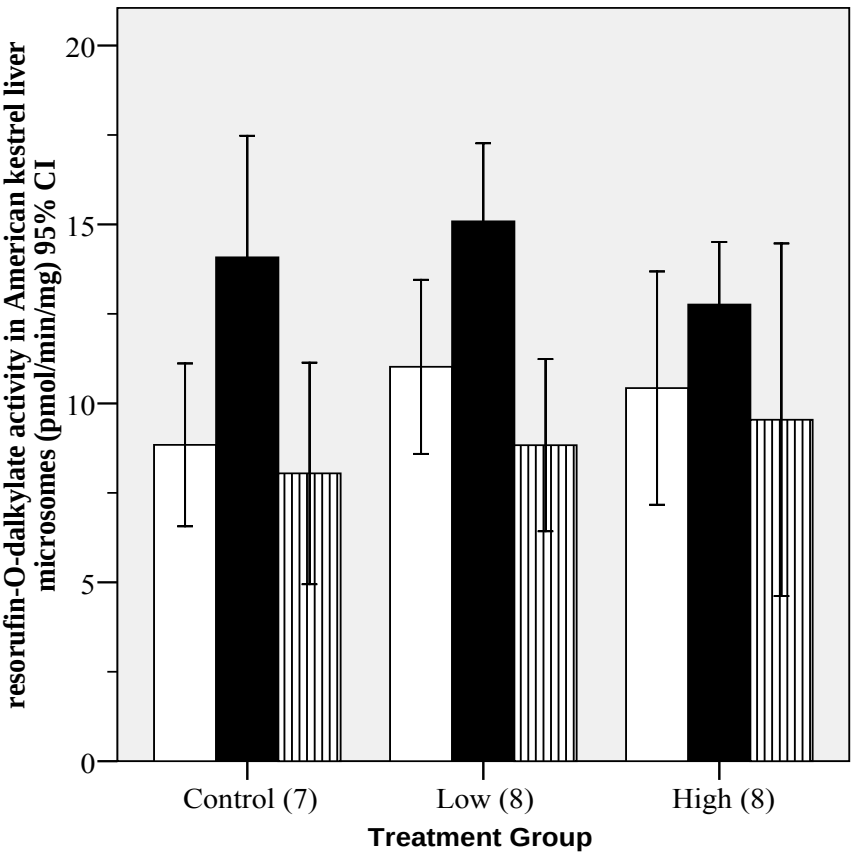

Figure 4-3 Ethoxyresorufin (EROD, CYP1A1) (light bars) (p = 0.413), methoxyresorufin (MROD, CYP1A) (dark bars) (p = 0.798), and benzyloxyresorufin (BROD, CYP2B) (hatched bars) (p = 0.279) O-dealkylase activities in liver microsomes of American kestrels exposed to breathing grade air (control), low-dose (benzene-0.1 ppm, toluene-0.8 ppm), and high-dose (benzene-10 ppm and toluene-80 ppm) in 2006. Horizontal bars represent the mean O-dealkylase activity. The number of individuals per exposure group is presented in parenthesis.

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4.3.3 Circulating Immature Red Blood Cells

Year 1

There was no significant difference between control and high-dose birds with respect to immature RBCs in the systemic circulation ($p = 0.564$) (Figure 4-4).

Year 2

One way analysis of variance (ANOVA) showed a significant difference in circulating polychromatophils between dose groups ($F_{2, 20} = 4.183$, $p = 0.032$). Bonferroni post hoc comparisons revealed fewer polychromatophils in the circulation of low-dose birds compared to control birds ($p = 0.029$) (Figure 4-5 a).

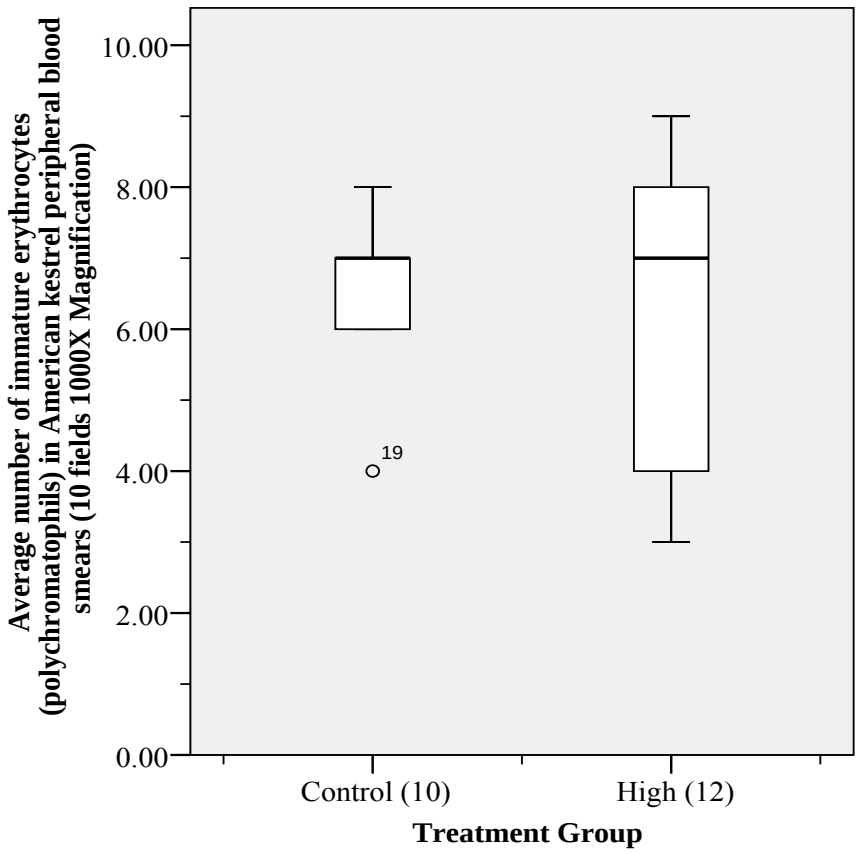

Figure 4-4 Polychromatophils in the systemic circulation of American kestrels exposed to breathing grade air (control) and high-dose (benzene-10 ppm and toluene-80 ppm) in 2005 (p = 0.564). Horizontal bars represent the mean number of polychromatophils per 1000 X field for each dose group. Open circles depict outliers. The number of individuals per exposure group is presented in parenthesis.

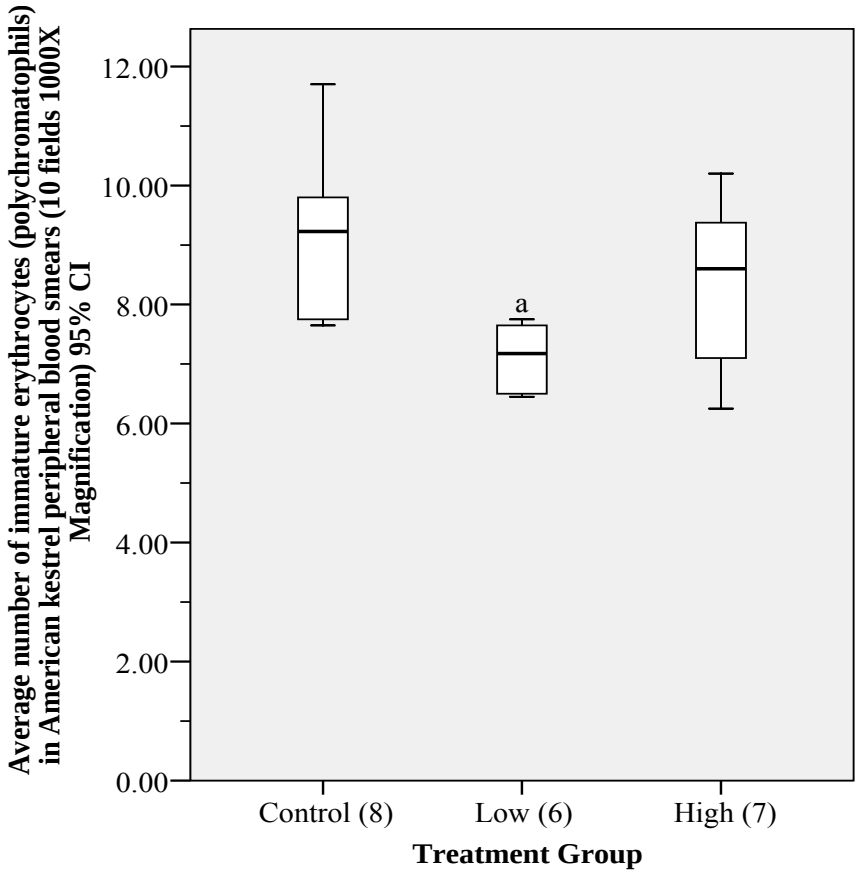

Figure 4-5 Polychromatophils in the systemic circulation of American kestrels exposed to breathing grade air (control), low-dose (benzene-0.1 ppm, toluene-0.8 ppm), and high-dose (benzene-10 ppm and toluene-80 ppm) ($p = 0.032$) in 2006. Horizontal bars represent the mean per 1000 X field for each dose group. Open circles depict outliers. The significant difference is indicated by a ($p = 0.029$). The number of individuals per exposure group is presented in parenthesis.

4.3.4 Bone Marrow Erythroid Lineage Cytology

Year 2

To evaluate the relationship between the pre- and post-mitotic pools of immature RBCs in the marrow and the marrow population with the circulating immature RBCs simple linear regression was used. In all exposure groups the number of mitotic cells significantly predicted the number of post-mitotic erythroid cells. Table 4-2 presents the importance of the effect (R^2), constant (B), and intensity of the effect (slope) values as well as the significance of the regression. Pearson 2 tailed correlations (p values) were used to describe the strength of the association between mitotic, postmitotic, and systemic erythroid cells and highlight the importance of the relationship between cell production in the bone marrow and systemic populations.

The control birds the mitotic pool was significantly correlated with the post-mitotic pool ($R^2 = 0.531$, $p = 0.040$), a relationship which does not change with exposure to high ($R^2 = 0.730$, $p = 0.014$) or low ($R^2 = 0.626$, $p = 0.019$) doses of benzene and toluene (Figure 4-6).

When the same regression model was used to predict the effect of the post-mitotic polychromatophils on the circulating polychromatophils, the relationship was lost in birds exposed to benzene and toluene. In control birds the number of polychromatophils in the marrow werecorrelated with those in ciculation ($R^2 = 0.667$, $p = 0.013$). In low ($R^2 = 0.180$, $p = 0.402$) and high ($R^2 = 0.000$, $p = 0.974$) dose birds the relationship was no longer significant (Figure 4-7).

Table 4-1 Descriptive statistics of the unstandardized and standardized coefficients used to calculate the linear regression for the relationship between bone marrow erythroid progenitor cells (mitotic and postmitotic) and immature red blood cells (polychromatophils) in the systemic circulation of American kestrels exposed to breathing grade air, benzene, and toluene in 2006.

Model	Slope (m)	B constant (b)	β	R^2	t	Significant linear regression (p)
1[a]	-0.737	322.612	-0.729	0.531	-0.261	0.04
1[b]	-1.005	377.634	-0.791	0.626	-3.166	0.019
1[c]	-1.024	361.293	-0.855	0.73	-3.679	0.014
2[a]	0.025	4.36	0.816	0.667	3.463	0.013
2[b]	0.007	5.661	0.424	0.18	0.936	0.402
2[c]	0	8.195	0.015	0	0.034	0.974

1 post-mitotic erythroid numbers are dependent on mitotic erythroid cell numbers (independent)

2 circulating immature rbcs are dependent on the post-mitotic polychromatophil numbers (independent)

[a] Control birds

[b] Low exposure (0.1 ppm benzene, 0.8 ppm toluene) birds

[c] High exposure (10 ppm benzene, 80 ppm toluene) birds

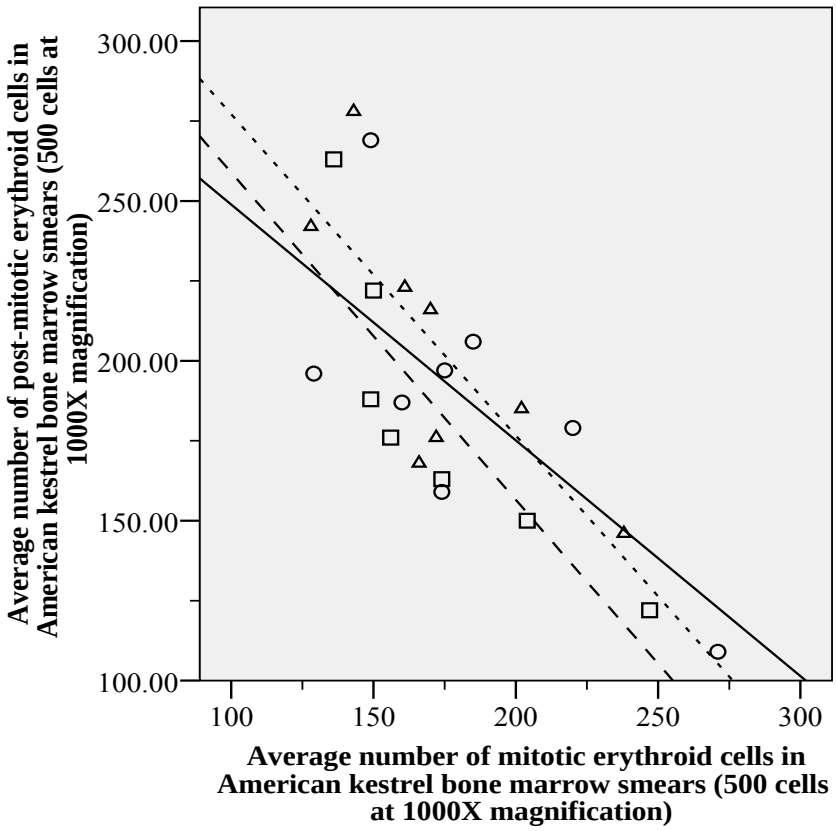

Figure 4-6 Relationship between erythroid cells of the mitotic pool and post-mitotic pool in the bone marrow of American kestrels exposed to breathing grade air (control), low-dose (benzene-0.1 ppm, toluene-0.8 ppm), and high-dose (benzene-10 ppm and toluene-80 ppm) in 2006, N = 23. The open circles and full line depict the relationship in control birds $R^2 = 0.531$, open triangles and small hatched line the relationship in low dose birds $R^2 = 0.626$, and the open squares and large hatched line represents the relationship in high exposure birds $R^2 = 0.730$.

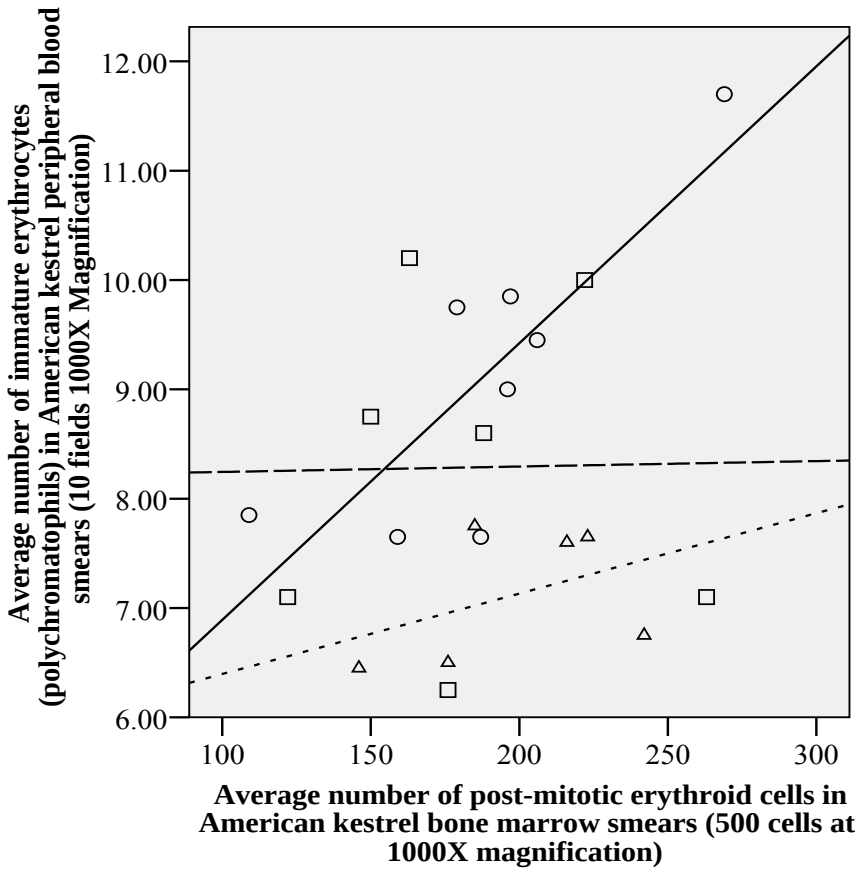

Figure 4-7 Relationship between immature erythrocytes in the post-mitotic pool in bone marrow and circulating immature erythrocytes of American kestrels exposed to breathing grade air (control), low-dose (benzene-0.1 ppm, toluene-0.8 ppm), and high-dose (benzene-10 ppm and toluene-80 ppm) in 2006, N = 21. The open circles and full line depict the relationship in control birds $R^2 = 0.667$, open triangles and small hatched line the relationship in low dose birds $R^2 = 0.180$, and the open squares and large hatched line represents the relationship in high exposure birds $R^2 = 0.000234$.

4.3.5 Behavioural Alterations

Year 1

There was a trend towards increased head bobbing in wild birds in the high exposure group compared to controls ($F_{1, 20} = 3.905$, $p = 0.063$) (Figure 4-8 a). Kruskal - Wallis analysis showed a significant decrease in the number of wing beats in high dose individuals ($p = 0.025$) (Figure 4-9-1a), the same data is shown log transformed in Figure 4-9-2. There was no difference in number of tail bobs or vocalization.

Year 2

There were no significant differences in any behavioural endpoints studied in captive birds exposed to a live Great horned owl in 2006 apart from a trend of increased vocalizations in exposed birds ($F_{2,31} = 2.136$, $p = 0.136$). The Fisher's LSD test showed that the high exposure individuals vocalized more compared to control ($p = 0.086$) and low dose birds ($p = 0.084$) (Figure 4-10 a).

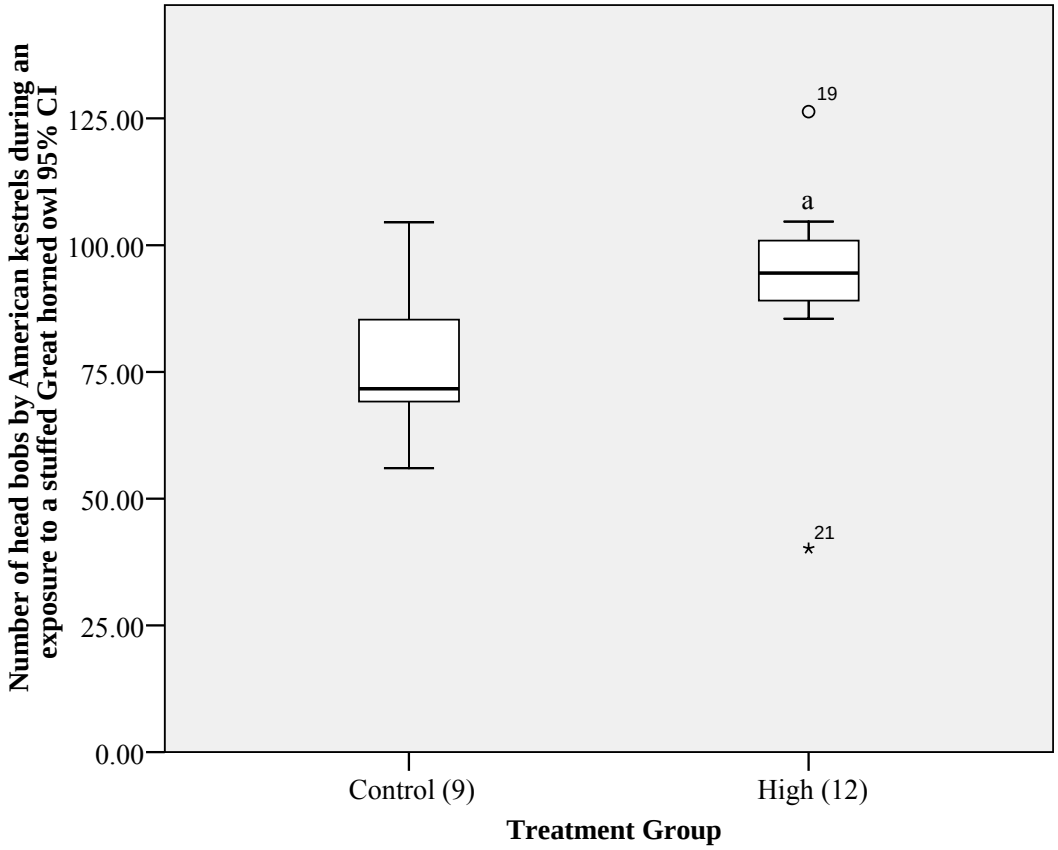

Figure 4-8 Number of head bobs as quantified by video analysis of American kestrels subjected to a stuffed Great horned owl after exposure to breathing grade air (control) and high-dose (benzene-10 ppm and toluene-80 ppm) in 2005. Horizontal bars represent the mean number of head bobs in 3 minutes for each dose group. Open circles and stars depict outliers. The trend towards an increased number of head bobs in birds exposed to high doses of benzene and toluene as compared to control individuals is shown by a ($p = 0.063$). The number of individuals per exposure group is presented in parenthesis.

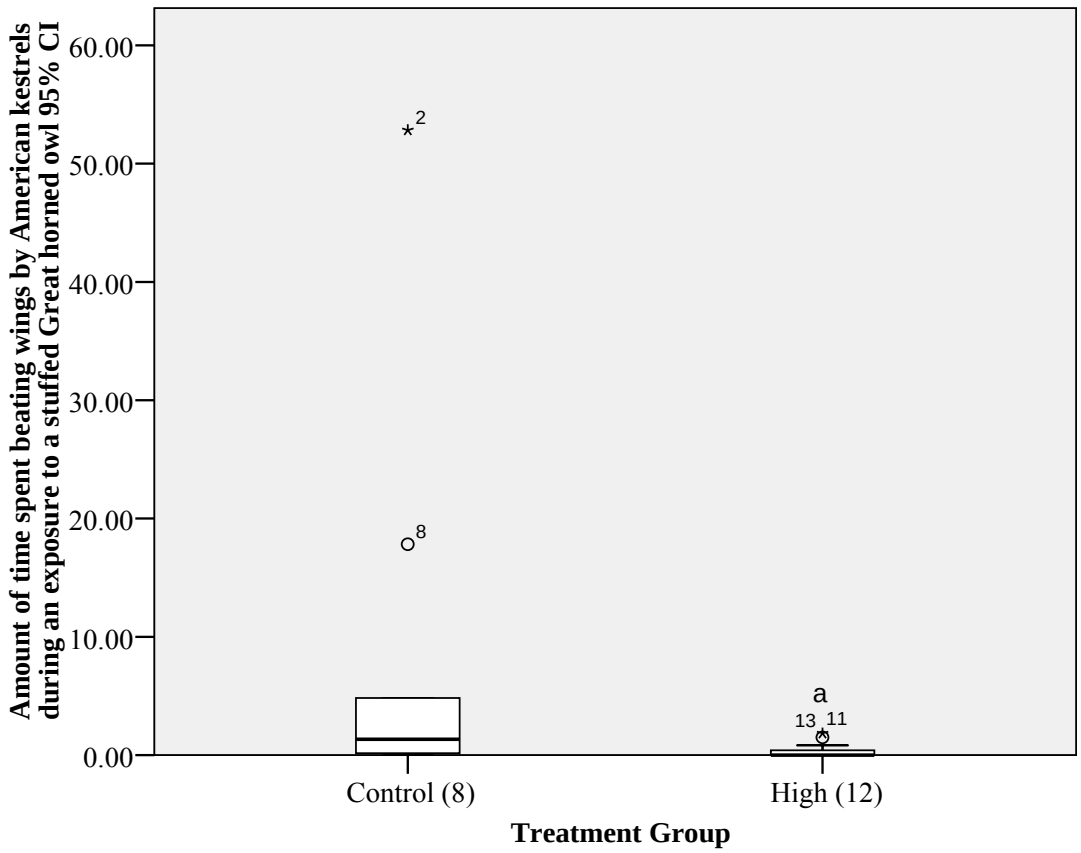

Figure 4-9-1 Amount of time spent wing beating (seconds) as quantified by video analysis of American kestrels being subjected to a stuffed Great horned owl after exposure to breathing grade air (control) and high-dose (benzene-10 ppm and toluene-80 ppm) in 2005. Horizontal bars represent the mean amount of time spent wing beating over 3 minutes for each dose group. Open circles and stars depict outliers. The significantly decreased amount of time spent wing beating in birds exposed to high doses of benzene and toluene as compared to control individuals is shown by a ($p = 0.025$). The number of individuals per exposure group is presented in parenthesis.

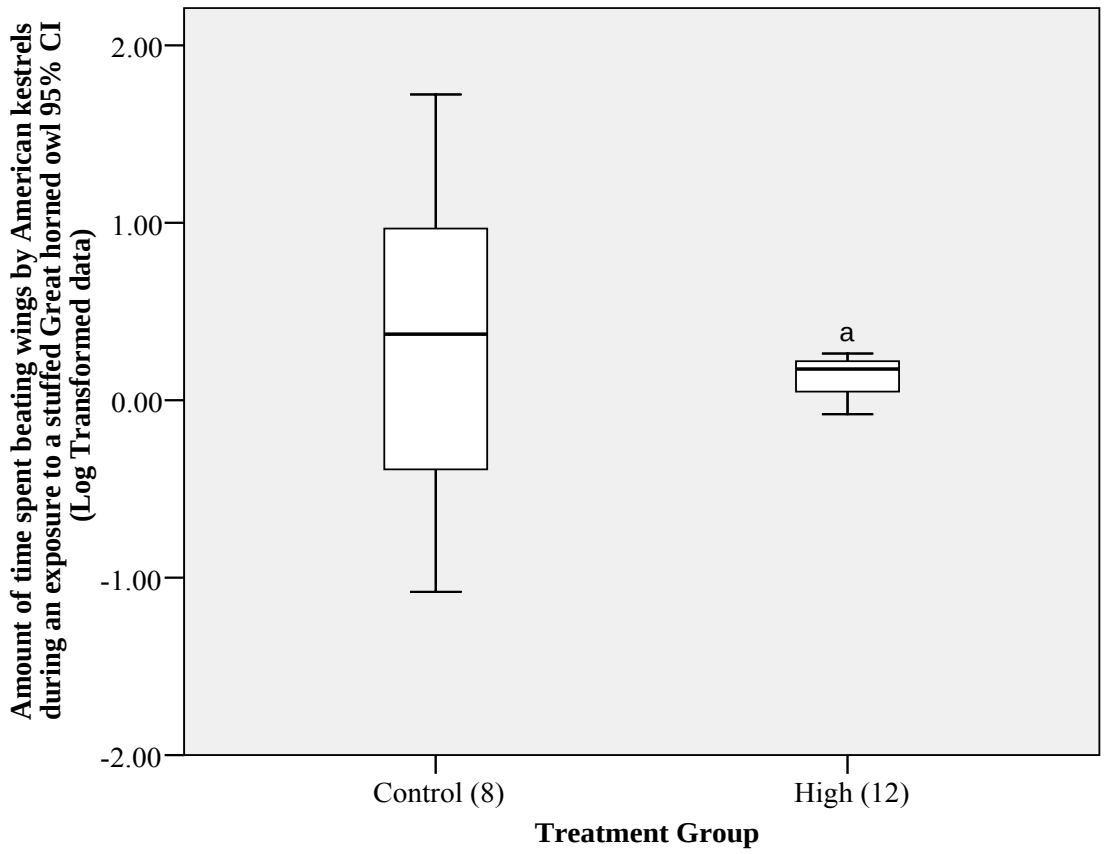

Figure 4-9-2 Log transformation of the amount of time spent wing beating (seconds) as quantified by video analysis of American kestrels being subjected to a stuffed Great horned owl after exposure to breathing grade air (control) and high-dose (benzene-10 ppm and toluene-80 ppm) in 2005. Horizontal bars represent the mean amount of time spent wing beating over 3 minutes for each dose group. Open circles depict outliers. The significantly decreased amount of time spent wing beating in birds exposed to high doses of benzene and toluene as compared to control individuals is shown by a ($p = 0.025$). The number of individuals per exposure group is presented in parenthesis.

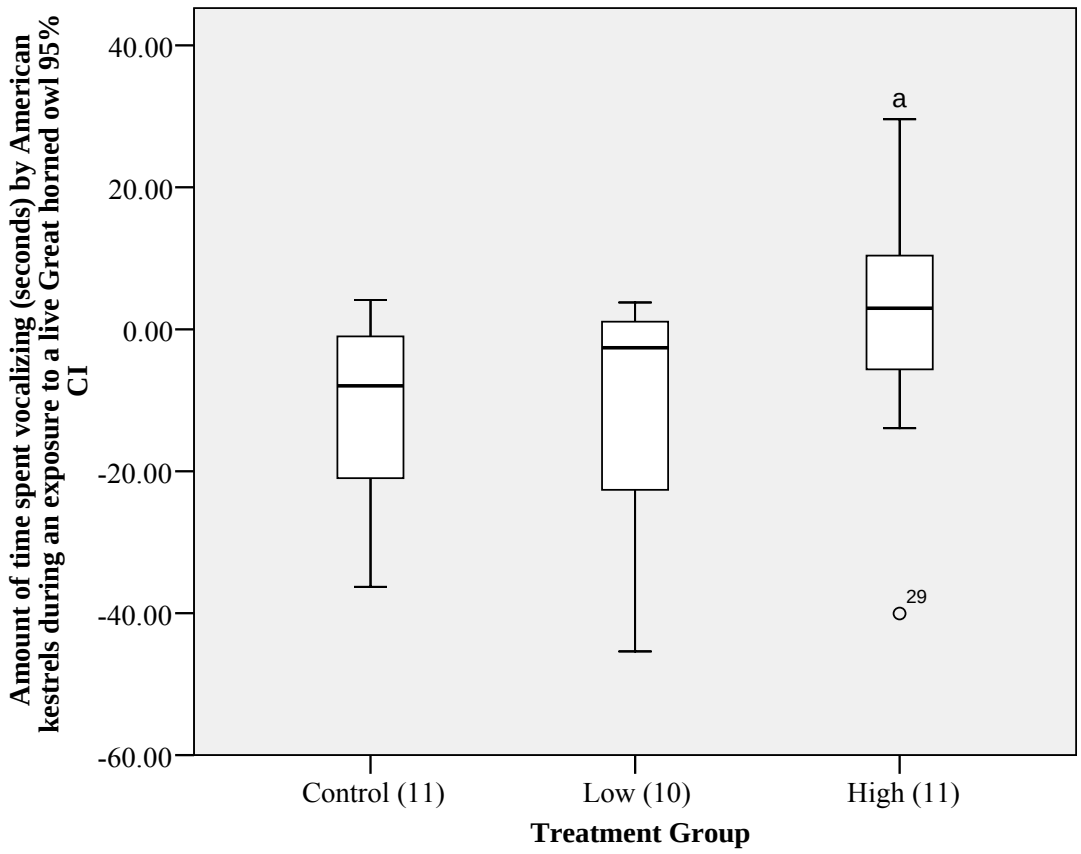

Figure 4-10 Amount of time spent vocalizing (seconds) as quantified by video analysis of American kestrels being subjected to a stuffed Great horned owl after exposure to breathing grade air (control), low-dose (benzene-0.1 ppm, toluene-0.8 ppm), and high-dose (benzene-10 ppm and toluene-80 ppm) ($p = 0.136$) in 2006. Horizontal bars represent the mean time spent vocalizing over 3 minutes. Open circles depict outliers. The trend towards an increased amount of time spent vocalizing in birds exposed to high doses of benzene and toluene as compared to control individuals is shown by a ($p = 0.086$). The number of individuals per exposure group is presented in parenthesis.

4.4 Discussion

Cytochrome P450 Enzyme Activities Years 1 and 2

The biotransformation capacity of liver microsomes is 5 times greater than lung microsomes and the products of metabolism are vastly different (Chaney and Carlson 1995). When benzene is the substrate, the lung will produce more quinone metabolites than will the liver. The situation is complicated by toluene's enzyme specific inhibition of CYP2E1 resulting in decreased biotransformation of both compounds, which in turn, would decrease haematotoxic and increase neurological effects (Andrews *et al.* 1977).

To quantify the enzyme activities of liver and lung CYP1A, 2B, and 1A1 resorufin product formation assays (MROD, BROD, and EROD) were performed. The results show no difference in any liver enzyme activity, but a dose dependent decrease in lung CYP1A activity was apparent in both low and high dose captive kestrels in 2006. In 2005, the lung microsome assays showed no activity in any assay so was un-evaluatable do to lack of sample, unlike the 2006 samples. Both 2005 and 2006 high dose groups showed no difference in liver biotransformation activities compared to controls. This suggests that CYP1A activity in the lungs of birds exposed to 10 ppm benzene and 80 pm toluene was inhibited to a greater degree than in low dose birds and therefore the formation of phenolic metabolites from benzene metabolism may have been impeded. The data presented here must be interpreted with a great deal of caution as the inhibition in the high dose group is a trend but on the same note the dose-dependent decrease as benzene/toluene concentrations increase is significant. Another consideration is the highly conservative nature of the statistical test (Mann-Whitney U) used to determine

statistical differences between the control and dosed groups, which may have under represented the inhibition.

Haematologcal Changes Years 1 and 2

Bone marrow cellularity and polychromatophils can both be used as indicators of systemic toxicity in response to exposure to many chemicals but by far the most commonly studied is benzene (Tunek *et al.* 1981; Plappert *et al.* 1994; Bernauer *et al.* 2000; Yoon *et al.* 2001; Lovern *et al.* 2001). To quantify benzene damage, bone marrow cellularity and related systemic components are routinely measured (Elmore 2006).

The high dose groups in both years showed no change in circulating polychromatophil numbers however the low dose group in year 2 clearly had fewer immature RBCs in circulation than would have been expected based upon control bird cell numbers. There are two main mechanisms by which an immature RBC pool could decrease in circulation. The first occurs when a surface protein is signaled to shorten the lifespan of the polychromatophilic erythrocyte, the second when the immature RBC matures more rapidly than would be expected and enters the mature erythrocyte pool (Harvey 2001). The data seems contradictory as the individuals exposed to high doses of benzene and toluene had the most pronounced loss of correlation between the post-mitotic pool and systemic pool of immature erythrocytes compared to the low and control dose birds. Therefore the data must be interpreted based on the loss of immature red blood cell numbers and the lack of correlation between the post-mitotic erythroid cells in the bone marrow and the circulation in low-dose birds.

Linear regression analysis indicates a loss of communication between the post-mitotic erythroid pool in the marrow and the number of circulating immature RBCs but a

direct correlation between mitotic and post-mitotic erythroid cell numbers. As the number of post-mitotic cells decreased, the mitotic cells increase in all dose groups (control, low, and high). The relationship between post-mitotic cells and systemic polychromatophils in low and high dose however was lost. In control birds, the number of immature erythrocytes increased as the post-mitotic pool of erythrocytes increased. Birds exposed to low doses of benzene and toluene show a much weaker relationship between the two compartments, and this relationship is virtually non-existent in high dose individuals. The data decidedly shows that the loss of communication or toxicity is not occurring in the mitotic or the post-mitotic erythroid bone marrow pool. Since the post-mitotic pool correlates to the mitotic pool there is no change in the maturation sequence within the marrow.

The relationship between this single cell type within the bone marrow, the circulation, and tissue compartments is not well understood and no data to explain this relationship could be found for birds. The lack of a dose response may be explained using the lengthy description in the previous section about the consequences of CYP2E1/1A inhibition. Briefly the levels of toluene (80 ppm) present in high-dose exposures may act as a buffer decreasing the metabolism of benzene and thusly the toxic metabolite products. The low-dose group may not have had the levels of toluene (0.8 ppm) present to bind CYP2E1 leading to increased metabolite formation and deleterious effects on the erythroid cell line. This theory is substantiated by the MROD data, which shows suppression of CYP1A in the high dose group

The bone marrow as a target for xenobiotic toxicity has not been well studied in avian species. The administration of the antihelmentics fenbendazole and albendazole

and the subsequent bone marrow hypoplasia have been studied in pigeons, doves, two species of vultures, and Maribou storks (Weber *et al.* 2002; Bonar *et al.* 2003). These studies do not provide any baseline cell numbers or activities in avian bone marrow samples, the results mainly focus on the granuloid and myeloid pools, and the chemical being studied has a very different mechanism of action as compared to benzene. A decreased number of polychromatophils could eventually lead to a state of anemia which in turn decreases the amount of oxygen available to the body which could have detrimental consequences in a wild bird attempting to catch prey or avoid a predator.

These results must be interpreted assuming that benzene targets the bone marrow and exerts its major toxic effect at the progenitor cell level (Morgan and Alvares 2005). The current study has shown for the first time a bone marrow evaluation after exposure to benzene and toluene in a falconiform species. It describes the subsequent loss of communication between the post-mitotic cells of the bone marrow and the same cells within the systemic circulation. If we take into consideration the relatively short study duration, non-typical species model (non-mammalian), extremely low exposure dose showing alterations (benzene 0.1 ppm), and that the conservation of the target organ the results presented here are indicative of unfavourable conditions occurring in the red blood cell lineage subsequent to benzene and toluene exposure.

Behavioural Alterations Years 1 and 2

Behavioural testing of wildlife species is a relatively under utilized field as compared to laboratory rodents which are commonly used for testing behavioural effects associated with toxicant exposure (Tarantino and Bucan 2000). Balgooyen (1976) was integral in observing and characterizing various foraging, mating, and defensive

behaviours of American kestrels in the wild. To determine if any toxicity was occurring due to toluene exposure a battery of tests designed to quantify aggression (vocalization, wing beating), balance (tail bob), and ability to focus (head bobs) was implemented (Balgooyen 1976; Gard *et al.* 1989).

Behavioural analysis revealed an increased number of head bobs and decreased amount of wing beating in 2005 and a trend towards an increased amount of time spent vocalizing in 2006. The behavioural changes are again indicative of what would be expected when benzene and toluene are simultaneously administered but the results are not statistically strong as there were many discrepencies between years 1 and 2.

A possible theory is that the increased vocalizations, head bobs, and decreased wing beating in high exposure individuals is consistent with the greater degree of inhibition of CYP1A, as they are behavioural alterations. The decreased biotransformation leads to increased levels of toluene available for transport to the brain, where it can cause dopamine neurotransmitter dysfunctions leading to memory and spatial behaviour defects (Voneuler *et al.* 1993). The low dose group in contrast exhibits classic dysfunction of the red blood cell line as a byproduct of benzene's increased metabolism due to a lack of inhibition of CYP1A (Cronkite *et al.* 1989; Yoon *et al.* 2002; Snyder 2004), with no signs of behavioural dysfunction.

Two major discrepancies between year 1 and 2 were unavoidable and could explain differences in altered aggressive behaviours between 2005 (head bobs, wing beating) and 2006 (vocalization) individuals. In 2005 a stuffed Great horned owl (GHO) was used in lieu of a live animal. Also in 2005 the predator was placed above the kestrel rope perch and in 2006 the predator was on the ground below the perch. These 2

differences could evoke very different behaviours from kestrels due to perceived threat. American kestrels do not react similarly to live and mounted GHOs (Gard *et al.* 1989) making the results between years 1 and 2 are difficult to interpret. The level of aggression as measured by vocalization time and wing beating showed a decrease in wing beating in 2005 and increased vocalization in 2006. It is has been documented that American kestrels view GHOs as predators and that behavioural responses are less aggressive towards taxidermic mounts than live animals (Gard *et al.* 1989; Gard and Bird 1990) . The decreased aggression towards the mounted GHO in addition to possible previous interactions with wild GHOs of wild American kestrels (2005) would be very different from captive kestrels (2006) exposed to a live GHO. The less intense response in 2005 is likely explained by using a mount. The 2006 captive kestrel data presented here agrees with the previous findings of Gard et al (1989). In any case there are behavioural differences between high dose kestrel as compared to low and control exposure groups.

A show of dominance in the form of increased vocal output or wing beating to give the illusion of increased size may scare off a live predator leading to a successful brood year, increased food availability, or access to prime breeding territory. In contrast increased aggressiveness towards perceived threats that normally occur during the breeding season (including the nestling period) could also pose a risk to chicks. Injuries by talons during nest exiting or intentional aggression during times of increased stress (weather, food availability) towards nestlings have been observed (personal observations). There are definite benefits and pitfalls of increased aggression.

Toluene is known to accumulate in the white matter of the brain where, in a bird, it will interrupt the action of dopaminergic receptors affecting functions related to vision

and balance (Voneuler *et al.* 1993). To measure the kestrels' ability to focus the number of head bobs was quantified and balance was assessed by tail bobs. The only difference was an increased number of head bobs in high dose individuals in 2005, which was not seen in 2006 as was expected. The effect of placement of the owl on the floor vs. higher up in the room could draw the conclusion that it is easier for a kestrel to focus but there is no scientific evidence to validate this theory. The differences in behaviour are most likely explained by the mount vs. live GHO and captive vs. wild bird differences between 2005 and 2006.

4.5 Conclusion

American kestrels nesting in close proximity to areas with increased concentrations of benzene and toluene could be at an increased risk for haematological problems with respect to polychromatophil numbers and neurological defects manifesting as altered aggression responses and inability to focus. Toluene will act as an inhibiter of benzene's metabolism decreasing toxic effects on red blood cells while at the same time increasing behavioural effects in a high dose situation. When toluene is not present in sufficient concentrations the neurological effects will be null and the erythroid line will be targeted.

In order to properly interpret the haematological and behavioural data it is necessary to generate data for the primary biotransformation enzyme, CYP2E1 (Snyder and Hedli 1996; Gut *et al.* 1996b; Powley and Carlson 2000; Powley and Carlson 2001; Yoon *et al.* 2002). Liver and lung microsomes are scheduled to be analyzed using a specific CYP2E1 substrate, chlorzoxazone, to measure the activity by quantification of 6-OH chlorzoxazone product formation by HPLC photo-diode array (PDA) analysis (Court *et al.* 1997).

Future analysis with wild birds should focus on more sensitive enzyme activity assays, specifically those measuring the primary enzyme involved, CYP2E1. Exposures to different chemicals associated with flared gas in a variety of combinations and field studies of birds in these high exposure areas would also increase the available data with respect to wild birds and allow the derivation of avian specific safety levels for various environmental pollutants.

CHAPTER 5

GENERAL DISCUSSION

Variables such as hepatic and pulmonary enzyme induction, and immune, endocrine, and haematological biomarkers have often been used in conjunction to assess the biological effects associated with contaminant exposure in avian species (Briggs *et al.* 1997; Brunstrom and Halldin 1998; Dawson 2000; Smits *et al.* 2002; Fernie *et al.* 2005b). Neither benzene nor toluene have been well studied in birds despite their presence in all ambient air samples (urban and rural locations) with higher than background levels being found in high oil and gas activity areas (benzene - 0.08 ppm, toluene - 0.08 ppm) (Carmalt 2005). The LOAEL for immunological but more-so haematological effects (decreased lymphocyte counts) in mammals exposed to benzene is 10 ppm (Rozen *et al.* 1984a). This level has been significantly re-evaluated and decreased to reach the 0.009 ppm reference concentration (RfC) for allowable daily exposure (Integrated Risk Information System 2003). The 80 ppm LOAEL for neurological effects (dopamine receptor dysfunction) associated with exposure to toluene (Voneuler *et al.* 1993) shows it is less toxic than benzene and the much higher allowable exposure limit (RfC), 1.33 ppm, reflects this (Integrated Risk Information System 2005). The current study is based on a non-mammalian model – the American kestrel, in an attempt to the discern the differences in metabolism and subsequent effects on the immune, endocrine, haematological, and neurological systems of birds as compared to mammals. As stated,

by Carmalt 2005 the levels of benzene in air samples taken near flare stacks in Alberta and Saskatchewan exceed the allowable daily exposure levels while toluene is within an acceptable range. If we consider the overly high benzene levels on the prairies which are 10 times greater than the acceptable exposure limit, and the fact that benzene and toluene RfC values were calculated in rodents that do not share the same respiratory physiology or enzyme capabilities as birds, there is a strong argument for re-evaluating the LOAELs used to derive exposure limits (RfCs) for both chemicals, to protect birds as well as mammals at risk.

Birds exposed to doses of benzene and toluene that were 100 times lower than the rodent LOAELs and 10 times higher than environmental levels (benzene - 0.1 ppm, toluene - 0.8 ppm) exhibited immunomodulation of the humoral (increased) and cell-mediated (decreased) systems, as well as a marked reduction in the number of circulating polychromatophils, a loss of correlation between the post-mitotic pool of polychromatophils in the bone marrow and in circulation, and a small degree of pulmonary CYP1A enzyme suppression. There was also an eosonophilia, basophilia, and to a lesser degree monocytophilia present. Some of the above defects are well documented affects associated with benzene exposures (Snyder *et al.* 1993; Yoon *et al.* 2001; Morgan and Alvares 2005), others are mre difficult to interpret. The only effect that would be attributed to toluene exposure, behavioural alteration, was not evident in low-dose individuals which may be due to the inhibitory effect toluene has on benzenes metabolism (Andrews *et al.* 1977).

Mammalian LOAELs for benzene and toluene were used, as a comparative tool to determine if the United States Environmental Protection Agency (US EPA) calculated

RfC values would be protective for wild birds. Mammals exposed to 10 ppm benzene presented with decreased lymphocyte counts and toluene exposures of 80 ppm have manifested as dopamine receptor dysfunction. To evaluate the effects in birds, similar endpoints were measured. American kestrels in high-dose exposure situations presented with the same immunomodulation as low-dose individuals (increased humoral immunity, and decreased cell-mediated immunity). The cell mediated decreases however were much more dramatic**.** The serum retinol levels in these same birds were significantly lower after the entire dosing period as compared to levels at the beginning of the study. In addition there was an increase in the granulocytic maturation index (number of post-mitotic granulocyte cells in the marrow). Behavioural testing alluded to an increased aggression response in captive and decreased aggression response and inability to focus in wild kestrels. The most meaningful finding was the inhibition of CYP1A in lung microsomes. This inhibition may explain the behavioural effects in high-dose individuals as compared to low-dose. There likely was a high enough concentration of toluene to significantly inhibit benzenes and its own metabolism leading to decreased haematological and increased neurological effects. The findings of this study were similar to previous mammalian studies that present immune system defects, enzyme inhibition, and behavioural alterations (Andrews *et al.* 1977; Sato and Nakajima 1979; Purcell *et al.* 1990b; Medinsky *et al.* 1994b). Whether the effects on birds were more pronounced than in mammals is difficult to determine as different lab tests, cell types, and endpoints were used to evaluate the toxicity. It is sufficient to say there is a degree of toxicity in American kestrels that corresponds with other scientific studies in mammals after similar exposures to benzene and toluene.

If we compare the ratio of benzene to toluene in the test study (1:8) with the field situation (1:1) it may be inferred that the current study was inherently designed to decrease the toxicity attributed to benzene. The ratio of benzene to toluene is important in determining the toxicity of any exposure where both compounds are present. In order to compare the low-and high-dose data and determine a possible dose-response relationship it was imperative that the ratio be kept the same between both test groups. Further studies should address this and test the compounds in a 1:1 ratio to compare the range of effects. In addition it can be presumed that the same or more severe haematological effects could occur in birds exposed in nature, as the toluene level/ratio is lower on the Saskatchewan prairies. The low-dose levels used here are roughly 10 times higher than those measured in birds in nature by Carmalt in 2005 and were chosen to represent a worst-case scenario.

With respect to the various endpoints used to evaluate the toxicity associated with exposure to benzene and toluene in American kestrels some were more biologically meaningful and scientifically sound (Table 6-1).

Variable	Analysis Technique	Sample	Significance (p value)		Alteration/Response		Dose Group Affected
Immune System			2005	2006	2005	2006	
Humoral (primary, secondary)	ELISA	Plasma	0.995, 0.449	0.051	No Change,	Increased	Low, High
Cell-mediated	DTH	*In vivo*	0.028	0.001	Decreased,	Decreased	Low, High
Granuloid progenitor cell index	Histology	Bone marrow	N/A	0.095	Not measured,	Increased	High
Circulating leukocyte concentrations (eosinophil, basophil)	Histology	Blood smear	N/A	0.032, 0.009	Not measured,	Increased	Low
Endocrine System							
Triiodothyronine	EIA	Plasma	0.171	0.887	No change,	No change	N/A
Thyroxine	EIA	Plasma	0.737	0.837	No change,	No change	N/A
Retinol	HPLC	Plasma	0.008	0.048	Decreased,	Decreased	High
Retinol Palmitate	HPLC	Liver	0.39	0.922	No change,	No change	N/A
Enzyme activity							
CYP2E1	Fluorescence-EROD	Liver microsomes	0.398	0.413	No change,	No change	N/A
CYP1A	Fluorescence-MROD	Liver microsomes	0.268	0.798	No change,	No change	N/A
CYP2B	Fluorescence-BROD	Liver microsomes	0.2	0.279	No change,	No change	N/A
CYP2E1	Fluorescence-EROD	Lung microsomes	N/A	N/A	No change,	No change	N/A
CYP1A	Fluorescence-MROD	Lung microsomes	N/A	0.094	Not measured,	Decreased	High
CYP2B	Fluorescence-BROD	Lung microsomes	N/A	N/A	Not measured,	Not measured	N/A
Haematology							
Circulating polychromatophil number	Histology	Blood Smear	0.564	0.032	No change,	Decreased	Low
Erythroid mitotic vs. post-mitotic pool correlation	Histology	Bone marrow	N/A	0.040, 0.014, 0.019	Not measured,	Correlation	All
Erythroid post-mitotic vs. circulation pool correlation	Histology	Bone marrow	N/A	0.013, 0.402, 0.974	Not measured,	No correlation	Low, High
Behaviour/Neurological system							
Aggression (vocal, wingbeat)	Visual Quantitation	*In vivo*	0.544, 0.025	0.086, 0.590	Decreased,	Increased	High, High
Balance	Visual Quantitation	*In vivo*	N/A	0.53	Not measured,	No Change	N/A
Focus	Visual Quantitation	*In vivo*	0.063	0.775	No change,	Increased	High

Table 5-1. Comparison of endpoints measured, level of significance, and dose group affected in samples obtained from American kestrels exposed to benzene and toluene.

The immune system, the most reliable endpoint, has long been used as an important indicator of sub clinical toxicity in birds associated with exposure to environmental contaminants (Grasman *et al.* 1996b; Smits and Williams 1999; Smits and Bortolotti 2001; Smits *et al.* 2002; Fairbrother *et al.* 2004; Smits and Baos 2005; Fernie *et al.* 2005a). The techniques implemented here; the use of DNP-KLH as an immunizing agent and the subsequent measurement of antibody levels and delayed type hypersensitivity reactions, have been published in various peer reviewed journals and are possibly some of the most well studied with respect to birds (Smits and Bortolotti 2001;Fairbrother *et al.* 2004;Smits and Baos 2005). Having said this it is crucial that all birds used for a study have a naïve immune system with respect to the vaccine agent as over immunization may result in unreliable and highly variable responses. This was the only constraint of the study with respect to the immune system function and validity of the tests implemented.

The second most reliable variable studied was the blood system. It is difficult to study haematological endpoints in wild birds as there are few published reference standards and wild birds are highly variable. The strength of the evaluation of this system lies in the sheer number of granuloid and erythroid series variables evaluated (precursor and circulation cellular component evaluations). Two (blood, bone marrow) of the three major compartments (blood, bone marrow, tissue) were quantified presenting a picture of the partial functioning of the haematopoeitic system. The only misgiving was in the interpretation and animal numbers. The polychromatophil evaluation was not done in a blind study or by a clinical hematologist lending the results to scrutiny. Initial readings were compared with a lab certified clinical providing a solid foundation but not infallible

results. Other major misgivings were the lack of a standard other than control birds, the third compartment, tissue, was not evaluated, and only 23 birds were used for analysis. The amount of information generated made it possible to glimpse into the larger effects on the haematopoietic system and pinpoint an area of dysfunction, but not the cell type(s) targeted. It also produced inconsistent results that were difficult to interpret and many times were not statistically strong allowing much scrutinization. With all this said the fact remains this is the first ever American kestrel and more precisely avian specific data with respect to benzene and toluene exposures.

The key to behavioural testing is consistency. From year to year the protocol evolved making it difficult to compare the results from 2005 and 2006. The status of the birds was probably the largest effector. The differences in responses between captive and wild kestrels were different from year to year as shown by the lack of trends. The use of a mount vs. live GHO would also affect the results and decrease apparent trends. Keeping this in mind the behaviours analyzed, time of evaluation compared to total study duration, and species of predator was kept consistent making the results more reliable but not entirely conclusive. Differences that arose were simply due to the availability of better resources. In future studies a live predator should continue to be used and observations should be made with as little disruption to the bird as possible.

Enzyme activities with respect to the cytochrome P450 system are highly variable between species and individuals. To properly evaluate the toxicity of a chemical the proper isoform used for biotransformation must be identified in the species of concern. The current study shows that CYP1A in pulmonary microsomes was the most active form but this does not reflect the current literature in mammals (Chaney and Carlson 1995).

This may be a species specific difference that needs to be considered when deriving safety levels or a limitation of the method used here. The evaluation of induction or inhibition has been used in many species for many chemical evaluations through various exposure routes (Brunstrom and Halldin 1998; Wayland *et al.* 1998; Bishop *et al.* 1999; Gagnon and Holdway 2000). None of this literature identifies the effects of benzene in American kestrels. Therefore the technique used to measure the enzyme activity was sound (Papp *et al.* 2005a) but the effects of benzene and toluene in American kestrels after inhalation exposure was not well studied making it a stab in the dark with respect to the isoform(s) responsible for biotransformation of benzene and toluene in wild birds. In order to properly evaluate this system, which is integral, considering the mechanism of toxicity of these two chemicals, a more sensitive test is required. Currently an HPLC method to measure the activity of CYP2E1 using the biotransformation of chlozoxazone to 6-OH chlorzoxazone is being validated by our laboratory and will be used to analyze lung and liver microsomes from the study birds. A study comparing the activity of CYP1A and 2B using an HPLC method as compared to fluorescence detection should also be validated in bird samples to determine the most sensitive test.

Vitamin A and thyroid levels had not been evaluated in the current lab to the extent the immune system had. Vitamin A levels in the plasma and liver were analyzed using a new HPLC protocol. Thyroxine and triiodothyronine levels were evaluated using kits designed for human plasma. After many trial runs both HPLC methods and EIA kits produced reliable results. These samples were the first to be run so there were no in house levels for American kestrels to compare control birds to making it difficult to interpret the results as a clinical lab would. Measurements of endocrine status of wild birds and their

validity as sensitive biomarkers are becoming more prevalent in the literature and widely accepted as beneficial indicators of proper functioning within an organism ((Dawson 2000; Colborn 2002; Mcnabb *et al.* 2004; Decuypere *et al.* 2005; Fernie *et al.* 2005b). The results are interesting and solid but not as well validated in our laboratory as other biomarkers.

Very briefly the necropsy findings should be addressed. The weight of organs is a potential indicator of dysfunction that can be used to corroborate a biochemical alteration. They are also often used as covariates to control for differences in body size between animals. I also consider this endpoint to be the least reliable. Anyone who has ever tried to remove thyroid glands, adrenals, thymus, or other small organs can attest to this. The variability of removal from one individual to another is high and the data must be closely observed for outliers and rigorous notes taken on techniques and landmarks for excision for organs such as the heart, and note the number of organs removed if paired.

The larger concern after completing this study is the possible effect the concentrations and ratios of toluene and benzene in exposure mixtures have on the ensuing biological changes within the animal exposed. It appears that a low-dose exposure could prove equally toxic in comparison to a high exposure situation. This is theoretically based on the data presented here and should be studied in much more depth with the addition of more exposure concentrations in order to generate a proper dose response curve for comparison to the haematological data. The primary difference between high and low concentrations is which biological system is more greatly affected, neurological vs. haematological. It appears that the immune system is a general target and likely the most sensitive endpoint to study no matter what the concentration or ratio.

The U-shaped dose response curve was present in low dose exposures with respect to the haematological system whereas high dose did not exhibit the same pattern. With respect to environmental contaminants it is a rare phenomenon, but one that is that is typical of drugs and vitamins that have a low therapeutic index and act through hormesis (Zapponi and Marcello 2006). The concept of hormesis is very different than the situation presented here as the crux of hormesis is that at low-doses there is a beneficial aspect and at high-doses there is an ensuing toxicity. A good example of this phenomenon is alcohol consumption, at low-doses alcohol protects against coronary heart disease and stroke but at high-doses it is carcinogenic and causes liver cirrhosis (Casarett and Doull 2001).With this being said**,** most aspects of hematology; the number of polychromatophils, heterophil: lymphocyte ratio, and numbers of eosinophils, basophils, and monocytes all presented a U-shaped dose response curve (Figure B-1, B-2, B-3). It is integral to consider the shape of the dose response curve when calculating LOAEL, NOAEL, and other safety levels as different calculations and models are required to properly interpret data. Future studies should examine this relationship as it alludes to an increased toxicity associated with environmental levels of benzene and toluene.

Immunological, haematological, and behavioural changes seem to be the most sensitive and should be studied in more depth. Changes I would recommend for future research in this area would be; use of a larger number and variety of species of birds, repeat vaccination trials with naïve birds, validation of the existing immune data by histological and immunohistochemical analysis of the the wing web to identify the cell types present which would corroborate the measured decreased DTH response, as well as validation of the specific cytochrome P450 isoforms responsible for benzene/toluene

biotransformation and more sensitive detection methods. Bone marrow examinations should identify the cellular sub-types within the mitotic and post-mitotic pool allowing the researcher to verify the basophilia, eosinophilia, and monocytophilia and pinpoint the location of disruption and if it is truly occurring or just a random event. Behavioural testing would be difficult to conduct in the field but is a necessity. The placement of cameras near the nest box would allow researchers to quantify some behaviours such as head and tail bobbing, survival of young, and aggression towards young. Future work should also investigate the toxicity of other VOCs and air pollutants in complex mixtures and their biological effects in an avian model and the applicability of the data to larger falcons in a field setting.

In summary, birds exposed to benzene and toluene in the laboratory situation show marked similarities (T cell sensitivity) and differences (U-shaped haematopoietic response and vitamin A alterations) to mammals used in similar study designs. The current study shows that there are important biological, life histories, and previous chemical exposure differences between wild and captive American kestrels that must be considered when extrapolating data. With respect to current RfC levels, rodent LOAELS, and benzene and toluene levels measured on the prairies, raptors may be at an increased risk for immunological, endocrine, and haematological defects. The sensitivity of birds may be a product of the enzyme capabilities of the avian lung which shows a stronger response in kestrels than in mammals (Kallas 2004) and likely played an integral role in the ensuing altered biological responses witnessed here after benzene and toluene exposures. These alterations illustrate the need for avian specific data to determine allowable exposure concentrations rather than calculations based on mammalian data.

CHAPTER 6

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APPENDIX A

STRUCTURAL COMPONENTS, DESCRIPTION OF CONSTRUCTION, AND SAFETY TESTING OF THE ENVIRONMENTAL INHALATION CHAMBER USED TO EXPOSE AMERICAN KESTRELS TO BENZENE AND TOLUENE.

Inlet System for Inhalation Exposure Chamber

Aluminum cylinders containing the benzene and toluene test air were connected to the inlet gas line by a Re 212 dual stage chrome-plated forged brass body five port configuration, 316 L stainless steel diaphragm regulator with a compressed gas association (CGA) 590 connection supplied by Praxair (PRX 212-3301-75-000, Praxair, Saskatoon, SK, Canada) (Figure 2.1). The regulator was modified by removing the relief valve and attaching a ¼'' X 3/8" stainless steel female internal pipe (FIP). Breathing grade air release was controlled by a CGA 346 Oxygen L-Tec regulator (150-540). The modified connection of the 212 regulator and the Oxygen L-Tec regulators attached to ¼" compression X 3/8" hose barb brass fittings (A&B Quality Welding Torch and Prop Repair, Dalmeny, SK, Canada) leading into a ¼" soft copper (Cu) gas line. The 2 separate ¼" gas lines delivering control or test gas were controlled by a 1/4" brass threaded ball valve by a brass ¼" national pipe thread (NPT) X ¼" compression. This allowed the separation of the control and contaminated gas lines (Figure 2-2). The ball valves were soldered to 1/4" soft Cu tubing by a ¼" coupler that merged the 2 gas lines at a ¼" brass compression T. The single soft Cu ¼" gas line emerging from the compression T was soldered to ½" soft Cu tubing by ¼" to 3/8" and 3/8" to ½" Cu couplers. The ½" soft Cu tubing was soldered to a brass 1/4" FIP X 5/8" by a ½" coupling terminating in a 1/8" X ¼" brass bushing. A 1/8" X close nipple was used to attach the 1/8" female NPT inlet connection of the 316 stainless steel 65-mm direct reading 0-10 LPM Gilmont flow meter (C-32014-17, Cole Parmer Canada Inc, Anjou, PC, Canada) to the inlet flow line. The flow meter outlet port screwed directly onto a 350 cc Salter 7600 bubble humidifier (Medigas Saskatoon, SK, Canada) by a plastic easy grip quad style wing nut. The 8 mm

plastic exit port barb of the humidifier was attached to a brass 1/8” X close nipple by ¼” plastic tubing and ring clamps (Figure 2-3). The close nipple connected to a 1/4” FIP X 5/8” ending in a ½” external diameter. The FIP was soldered to ½” hard copper pipe that bifurcated at a Cu T into two separate gas lines each connecting to a gas distribution hexagon to which the animal holding cylinders were attached. The gas line connected to the hexagon by a ½” compression coupling linked to a ¼” FIP X ½” Cu with a ¼” X close nipple that screwed onto the ¼” inlet port that was drilled into the top of the distribution hexagon.

Gas Distribution and Animal Holding Chambers

Two separate 10” X 5” hexagons were constructed out of 12 Gauge galvanized steel (12-G). A 3” circle was punched out of the middle of each side to allow attachment of the individual animal holding chambers (Bergen Industries, Drake, SK, Canada). All welded joints were constructed and pressure tested by a journeyman welder with a pressure ticket (TSL Mechanical Lanigan, SK, Canada). In order to attach the individual chambers a 3” X 5” galvanized steel PTF male nipple was cut on a 45 degree angle and welded over the punched out hole in the hexagon (Medallion Pipe Supply Company, Saskatoon, SK, Canada). Female internal threaded white 3”X 3” PVC couplers were screwed onto each of the 12 metal male nipples (Figure 2-4). The female couplers were attached with 3” PVC adapters to solid white 15” X 3” PVC pipe. Each of the 15” long PVC pipes was used as an animal holding chamber. 3” X ½” plywood perches covered with turf were placed 12 inches from the top opening of each cylinder (Figure 2.-5). A tap and dye set was then used to drill 12 - 3/8” holes in each of the 3” PVC caps that covered

the openings of the cylinders. A 3/8” brass male NPT hose barb was inserted into the cap holes (GreenLine, Saskatoon, SK, Canada) to allow attachment to the exhaust lines.

All PVC joints were sealed with non volatile compounds or silicone. Silicone was applied 3 weeks before the study start date to ensure the acetic acid had completely volatilized before the pilot study began. All welding joints were pressure tested, and any soldering was leak tested using soapy water and high pressure air while observing leaks in the form of bubbles.

Exhaust System

Attached to the chamber caps~ 28” of 3/8” diameter vinyl tubing (GreenLine, Saskatoon, SK, Canada) exhausted all expelled and excess air. The vinyl tubing from each of the 12 holding chambers merged at the 15” X 4” Black PVC filter tube (Figure 2-6). Three holes measuring 3/8” in diameter were tapped on the 4 quadrants of the filter tube and 3/8” brass male NPT hose barbs were inserted into the holes (Green line, Saskatoon, SK, Canada.) to carry gas from the chambers through a suspended 10 micron polypropylene felt filter bag with a plastic ring opening measuring 4 1/8” X 8” (PO5P3-PR, The Filter Factory Inc., Yuma, AZ, USA) filled with 1 lb of C-40 activated carbon (General Carbon, Paterson, N.J., USA). A 4” lid enclosed the filter while a 3/8” male NTP barb with vinyl tubing exhausted the filtered air to the exhaust flow meter. The exhaust line attached to the exhaust flow meter inlet port by a 3/8” brass male HPT hose valve with a 1/8” internal diameter. The exhaust port was attached to a 0.2 CFM, 15 volt vacuum pump (001-78164-00, Cole Parmer Canada Inc., Anjou, PC, Canada) that exhausted to a HEPA filtration system (2005) or a chemical biosafety hood (2006).

Activated carbon needed for the study was calculated using a specific software program (General Carbon, Paterson, N.J., USA), based on the molecular weights of benzene and toluene, concentrations used, the system flow rate, amount of carbon available, and the carbon's capacity to adsorb VOC of interest. The system required 0.0525 lbs (2005) or 0.053025 lbs (2006) of carbon to run for 24 hours a day for 30 days, continually. To evaluate the efficiency of the filter, the air downstream was monitored via a ppbRAE (Model PGM7240, ProRae Suite Software. U of S, Saskatoon, SK, Canada) for one dosing session (Table A-1).

Table A-1 ppbRAE measurements of total VOC content in ppb measured at the exhaust port of the acitivated C-40 filter prior to release to the HEPA filtration system.

Sample	Date	Time	Min(ppb)	Avg(ppb)	Max(ppb)
1	7/13/2005	14:17	413	1597	2683
2	7/13/2005	14:22	2683	3576	4285
3	7/13/2005	14:27	4285	4725	5088
4	7/13/2005	14:32	5071	5200	5350
5	7/13/2005	14:37	5192	5325	5462
6	7/13/2005	14:42	5424	5518	5607
7	7/13/2005	14:47	5557	5675	5798
8	7/13/2005	14:52	5726	5792	5874
9	7/13/2005	14:57	5786	5886	5955
10	7/13/2005	15:02	5781	5963	6060
11	7/13/2005	15:07	5791	5882	5979
12	7/13/2005	15:12	5862	5951	6050
13	7/13/2005	15:17	5843	5990	6110
14	7/13/2005	15:22	5989	6060	6141
15	7/13/2005	15:27	6024	6114	6215
16	7/13/2005	15:32	6072	6159	6270
17	7/13/2005	15:37	6067	6288	7456
18	7/13/2005	15:42	6992	7147	7475
19	7/13/2005	15:47	5352	7019	7206

Instrument: ppbRAE (PGM7240), Serial Number: 100367
Gas Name: Isobutylene, Last Calibration Time: 06/09/2005

APPENDIX B

U-SHAPED DOSE RESPONSE CURVE OF HAEMATOLOGICAL ENDPOINTS IN AMERICAN KESTRELS EXPOSED TO HIGH- AND LOW-DOSES OF BENZENE AND TOLUENE.

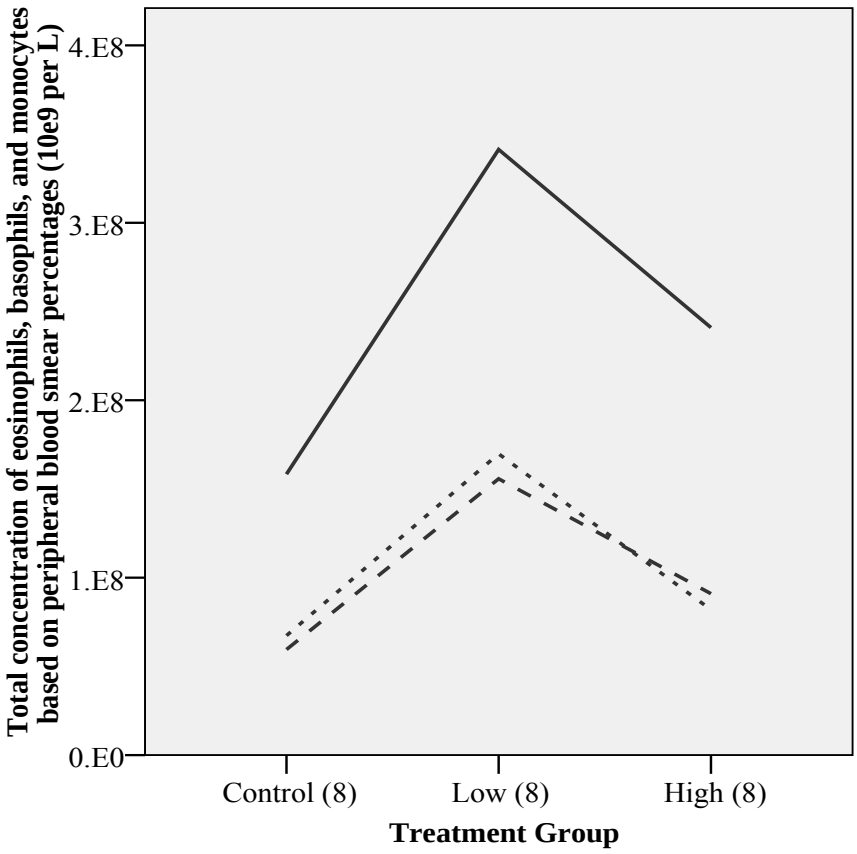

Figure B-1 Monocyte (full line), eosinophil (thin hatch line) and basophil (wide hatch line) concentrations (10^9 per L) in American kestrels exposed to breathing grade air (control), low-dose (benzene-0.1 ppm, toluene-0.8 ppm), and high-dose (benzene-10 ppm and toluene-80 ppm) in 2006. The number of individuals per exposure group is presented in parentheses.

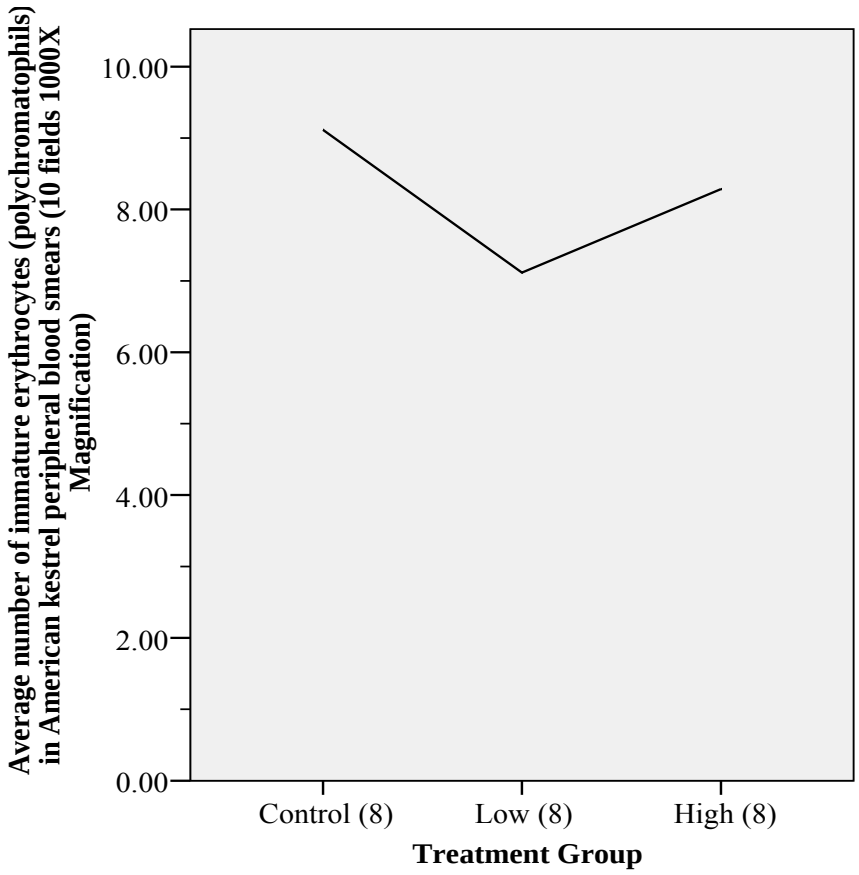

Figure B-2 Polychromatophils in the systemic circulation of American kestrels exposed to breathing grade air (control), low-dose (benzene-0.1 ppm, toluene-0.8 ppm), and high-dose (benzene-10 ppm and toluene-80 ppm) in 2006. The number of individuals per exposure group is presented in parenthesis.

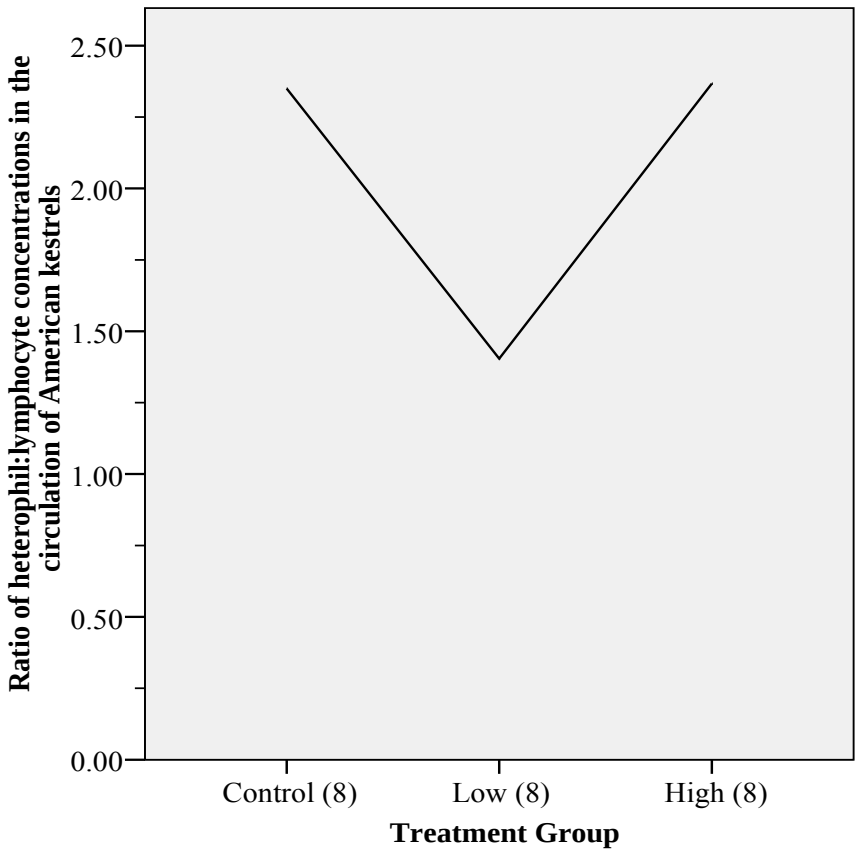

Figure B-3 Heterophil to lymphocyte ratio in the systemic circulation of American kestrels exposed to breathing grade air (control), low-dose (benzene-0.1 ppm, toluene-0.8 ppm), and high-dose (benzene-10 ppm and toluene-80 ppm) in 2006. The number of individuals per exposure group is presented in parenthesis.