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Use of Non-Destructive Biomakers to Measure Effects of Pesticide Exposure in Meadow Voles (*Microtus pennsylvaniacus*) Living in Golf Courses Ecosystems of the Ottawa/Gatineau Region

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USE OF NON-DESTRUCTIVE BIOMARKERS TO MEASURE EFFECTS OF PESTICIDE EXPOSURE IN MEADOW VOLES (MICROTUS PENNSYLVANICUS) LIVING IN GOLF COURSE ECOSYSTEMS OF THE OTTAWA/GATINEAU REGION

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ABSTRACT

Unlike the clear causal relationship between acute pesticide exposure and mortality in non-target wildlife, the association between sub-lethal pesticide exposure and chronic health problems (e.g., cancer, birth defects, endocrine disruption) are not as easily established. Studies that have reported relationships between pesticide exposure and chronic health issues are often surrounded by controversy and scientific debate. As a result of the uncertain relationship between pesticide exposure and human and wildlife health, and likely due to the direct alarm of their constituents, many municipal governments throughout Canada are implementing bylaws that regulate the use of pesticides. In some cases, pesticide use on public and private golf courses also falls under the newly created bylaws. One of the main arguments of those opposed to these restrictions is that there is a lack of scientific evidence linking pesticide exposure to poor human and environmental health. With municipalities planning pesticide control in order to protect human and environmental health, and with the controversial debate around pesticide exposure and the onset of health problems, it seemed timely to examine some of the potential effects of exposure to commonly used golf course pesticides.

The purpose of this doctoral thesis was to perform a detailed review of the carcinogenic and genotoxic potential of the most common pesticides used on golf courses of the Ottawa/Gatineau region, focusing on epidemiological and *in vivo* and *in vitro* mammalian laboratory evidence, and to conduct a non-lethal biomonitoring study to provide scientifically based measures of some possible consequences of repeated pesticide exposure on non-target animals living in areas of regular pesticide use. Specifically, genetic (e.g., DNA strand breaks and chromosomal damage), developmental (e.g., skeletal

birth defects and asymmetry) and organismal effects (e.g., changes in clinical haematology, body condition, blood parasite load) were assessed in meadow voles (*Microtus pennsylvanicus*) living in golf course ecosystems of the Ottawa/Gatineau region of Canada.

In general, voles from golf courses did not appear to differ in their asymmetry, skeletal birth defects, blood parameters, parasite load, body condition or level of chromosomal damage when compared to voles from reference sites. However, voles from golf courses did appear to experience significantly greater DNA damage expressed as single or double strand breaks. The comet assay showed that two parameters of DNA damage (e.g., tail length and moment) significantly decreased in relation to days since last application of a pesticide, and to days since the last application of a specific fungicide (Daconil®) containing a potentially genotoxic active ingredient (chlorothalonil). The slopes of these exponential curves were not significantly different than the half-life decay curve of chlorothalonil on vegetation (half-life data obtained from another study). Moreover, both parameters appeared to increase in a dose-dependent manner with the amount of the last application of Daconil®.

RÉSUMÉ

Contrairement au lien clairement établi entre l'exposition aiguë aux pesticides et la mortalité d'espèces non ciblées, la relation entre une exposition non mortelle et les maladies chroniques (cancer, anomalies congénitales, troubles endocriniens) est plus difficile à démontrer. Les études reliant les maladies chroniques à l'exposition de pesticides sont souvent controversés et source de débats d'ordre scientifique. À cause du lien incertain entre l'exposition aux pesticides et la santé humaine et animale, et vraisemblablement en réponse aux inquiétudes des citoyens, de nombreuses municipalités canadiennes ont mis en place des règlements restreignant l'utilisation de pesticides. Dans certains cas, les règlements municipaux incluent les terrains de golf, privés ou publics. Les opposants à de telles restrictions utilisent comme argument principal le manque de preuves scientifiques reliant l'exposition aux pesticides à l'appauvrissement de la santé humaine et de l'environnement.

Étant donné l'avènement des règlements municipaux et en raison des débats entourant l'utilisation des pesticides et leurs effets sur la santé, il semblait opportun d'examiner de plus près certains des effets potentiels reliés à l'utilisation de pesticides couramment utilisés sur les terrains de golf. Le but de cette thèse de doctorat est de mener un contrôle biologique afin de mesurer certains effets potentiels reliés à des applications non mortelles répétées de pesticides chez des espèces non ciblées. En particulier, l'impact sur la génétique (bris des brins d'ADN et dommage aux chromosomes), sur le développement (déformations squelettiques congénitales et asymétrie) et sur l'organisme (variations hématologiques, état de santé et présence de parasites sanguins) a été examiné

chez le campagnol des champs (*Microtus pennsylvanicus*) dans les écosystèmes de terrains de golf de la région d'Ottawa/Gatineau au Canada.

De façon générale, les campagnols en provenance des terrains de golf ne semblaient pas différents de ceux provenant des sites de référence, tant au niveau de leur asymétrie, des déformations congénitales, de leur hématologie, de la présence de parasites sanguins, de leur état de santé ou du degré de dommage aux chromosomes. Cependant, le dommage à l'ADN, se traduisant par des bris de brins simples ou doubles, a été plus important chez les individus en provenance des terrains de golf. Les deux paramètres exprimant le dommage à l'ADN, la longueur et le moment de la queue, ont diminué de façon significative, exponentiellement par rapport au nombre de jours depuis la dernière application de pesticides et en particulier par rapport aux nombre de jours depuis l'application du Daconil®, un fongicide contenant un ingrédient potentiellement génotoxique (le chlorothalonil). Les pentes de ces courbes exponentielles reflètent celle de la demi-vie du chlorothalonil sur les plantes. De plus, les deux paramètres semblaient augmenter selon la dose à la dernière application de Daconil®.

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University, Michigan State University, Cornell University, and the University of Idaho (http://ace.orst.edu/info/extoxnet). Many thanks also go to Genevieve Carr, Ian Scott, Steve Siciliano, and Adam Willis for their friendship, scholarly insight, field assistance and encouragement.

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GLOSSARY OF TERMS

These operational definitions are bolded at their first mention in the text

Biomarker: Biomarkers of anthropogenic stress are measurable changes in biochemical, physiological or behavioural states within cells, tissues, or whole individuals.

Carcinogenic: Cancer causing potential of a chemical.

Case-control Study: Epidemiological studies in which individuals that already exhibit a certain condition (e.g., cancer) are compared with individuals who do not, and researchers try to identify differences (e.g., pesticide exposure) between the two groups that may explain the condition.

Cohort Study: Epidemiological studies where groups of individuals with known differences (e.g., one cohort has been exposed to pesticides and the other has not) are followed over time and the occurrence of certain conditions (e.g., cancer) compared.

Comet assay: A biochemical assay used to quantify single and double DNA strand breaks and alkalie-labile sites.

Developmental Noise: Refers to a suite of processes that can lead to developmental instability; random differences in rates of cell division, growth and shape change, effects of thermal noise on enzymatic processes, and random differences in rates of physiological processes among cells.

Developmental Stability: The energetic contribution of an organism used to offset the stochastic effects of developmental noise that is generally achieved through negative feedback loops and hormonal regulation.

DMSO: dimethyl sulfoxide

Epigenetic: Used interchangeably with non-genotoxic, capacity of initiating any number of the steps in the onset of cancer without actually causing genetic damage (e.g., hyperplasia, cell proliferation).

Fluctuating Asymmetry: Random deviations from perfect bilateral symmetry with no genetic predisposition towards one side or the other being larger.

Genotoxic: Potential of a compound to cause direct DNA damage (e.g., base mutation, strand breaks).

Gy: Unit of absorbed dose of radiation, 1Gy is equivalent to 1 joule of energy being absorbed by 1 kg of tissue.

Hodgkin's disease: Cancer of the lymphatic system, specifically cancer of the immunocytes (B and T cells). Primarily from non-Hodgkin's lymphoma is the presence of Reed-Sternberg cells in tumours.

Hormesis: The stimulating effect of sub-lethal concentrations of a toxicant, generally to the benefit of the organism.

Immunosuppression: Inhibition of the body's immune system and its ability to fight infections or disease

Micronucleus: Fragments of chromosomes, or whole chromosomes, found in the cytoplasm. These pieces of DNA have not been incorporated into the nucleus during cell division as a result of genotoxic damage to stem cells.

Micronucleus assay: Toxicological test used to measure chromosomal damage (e.g., formation of micronuclei) in relation to genotoxic damage at the level of the stem cell.

Non-Hodgkin's Lymphoma: Cancer of the lymphatic system, specifically cancer of the immunocytes (B and T cells).

Odds Ratio: The comparison between the odds of exposure among cases to the odds of exposure among controls. The odds ratio is often used as an approximation of relative risk.

PBS: Phosphate Buffered Saline

Proportionate Mortality Ratio: The proportion of deaths due to a particular cause in an exposed population divided by the proportion of total deaths (or due to the disease of interest) in the unexposed population.

Precautionary Principle: The regulatory concept that lack of full scientific certainty should not be used as a reason for postponing measures to prevent environmental degradation.

Reproductive Status: A term to describe the sexual maturity (e.g., reproductive or non-reproductive).

Sarcoma: A form of cancer that arises in supportive tissue such as bone, cartilage, fat or muscle.

Tail length: A measure of the distance travelled by DNA fragments in the comet assay; the distance from the leading edge of the comet head to the leading edge of the tail.

Tail Moment: A measure of intensity of DNA damage in the comet assay; the tail ratio (i.e., the relative fluorescence intensity in the comet tail region, to that in the entire comet) multiplied by the distance (μm) between the centers of gravity of the head and tail regions.

CHAPTER 1:

GENERAL INTRODUCTION

INTRODUCTION

The causal relationship between acute pesticide exposure and mortality in non-target wildlife is well documented. In 1995-1996, over 20,000 Swainson's hawks (*Buteo swainsoni*) died in Argentina after they had fed on grasshoppers recently exposed to the insecticide monocrotophos (Woodbridge et al. 1995; Hooper et al. 1999). Similarly in the United States and Canada, American Wigeons (*Anas americana*) and Canada Geese (*Branta canadensis*) were found dead on golf courses after feeding on turf that had been treated with the insecticide diazinon (Frank et al. 1991; Kendall et al. 1992). In fact, mortality of wildlife species frequenting agricultural landscapes is thought to be a regular and frequent occurrence with the current suite of insecticides registered in North America (Mineau et al. 1999; Mineau 2002).

Unlike these clear causal relationships, the association between sub-lethal pesticide exposure and chronic health concerns (e.g., cancer, birth defects) are not as easily established. Studies that have reported relationships between pesticide exposure and chronic health issues are often surrounded by controversy and scientific debate. As a result of the uncertain relationship between pesticide exposure and human and wildlife health, and likely due to the direct alarm of their constituents, many municipal governments throughout Canada are implementing bylaws that regulate the use of pesticides. The objectives and specific details of these bylaws vary between municipalities but they all have the same underlying theme; bylaws are in place to regulate the public and private use of pesticides to protect the health of citizens and the environment (114957 Canada Ltée (Spraytech, Société d'arrosage) *v.* Hudson (Town) 2001 SCC 40)). One of the main arguments of those

opposed to pesticide use restrictions is that there is a lack of scientific evidence linking pesticide exposure to poor human and environmental health (Meech and English 2000).

PURPOSE OF THESIS

Considering the controversy and lack of scientific evidence regarding the effects of turf pesticide exposure that have lead to municipal pesticide control, it seemed timely to examine some of the potential organismal effects of exposure to commonly used pesticides. Accordingly, the purpose of this doctoral thesis was to 1) perform a detailed review of the carcinogenic and genotoxic potential of the most common pesticides used on golf courses of the Ottawa/Gatineau region, focusing on epidemiological and *in vivo* and *in vitro* mammalian laboratory evidence, and 2) to conduct a non-lethal biomonitoring study to provide scientifically based measures of some possible consequences of repeated pesticide exposure on non-target animals living in areas of regular pesticide use. Specifically, **genotoxic**, developmental and organismal effects of pesticide exposure were measured in meadow voles (*Microtus pennsylvanicus*) living in golf course ecosystems of the Ottawa/Gatineau region of Canada. I am unaware of any ongoing studies on the chronic health outcomes of animals (or people) that are likely to come into contact with pesticides on golf courses, even though there appears to be increasing potential for their unintentional exposure. An ancillary project was conducted during the course of this thesis to validate the use of one of the biomarkers (**fluctuating asymmetry**), and results of this study are presented in appendix IV.

GOLF COURSES and PESTICIDES

The game of golf developed approximately five hundred years ago in the medieval city of St. Andrews, Scotland, where it was played on ground designed and maintained by the forces of nature (Beard 1989). What is now referred to as the fairway and the green was then a "...linksland..." of many little pieces of connected land in a marsh that was composed of a "...fine leaf texture that was smooth and glassy when dry...", and today's sand traps and bunkers were originally burrows carved out of the land by sheep seeking shelter from the elements (Beard 1989). As the game gained popularity over the centuries (in fact, it was so popular during the Fifteenth and Sixteenth century that soldiers neglected their duties in order to play golf and the King of Scotland had to decree in 1457 and again in 1471 and 1491 that golf could not be played if it interfered with the good of the Realm (Beard 1989)) and spread across the world, the demand for lush, green, uniform turf has dramatically increased.

Before the Twentieth century, greens keepers employed weed pickers to remove unwanted plants, used unprocessed manure as turf fertilizer, sprayed nicotine as an insecticide, and utilized grazing cattle, sheep and rabbits to maintain a well clipped turf (Beard 1989). Present day golf course superintendents have many more pesticide products available to them to help maintain a well-groomed and healthy turf. A survey of golf courses in Alberta (n=33) by the Alberta Environmental Protection Office indicated that on average, almost 80 kg of active ingredient per year was applied (range between 14 and 167

kg). Of the products used, fungicides made up the majority (64%), followed by herbicides (34%) and insecticides (2%) (AEP 1998). As reported in a document published by the Attorney Generals Office of New York State, golf courses on Long Island used almost four to seven times the average amount of pesticides, on a kg/ha basis, than was used for agricultural purposes (Koppell 1994). With over 1600 golf courses in Canada (personal communication Royal Canadian Golf Association) and between 400 and 600 new courses created each year in Canada and the U.S.A., there appears to be increasing potential for unintentional human and animal exposure to turf pesticides. However, it is surprising that the majority of studies that have investigated the use of pesticides on golf courses have not focused on health issues. Rather, studies have focused on pesticide runoff, accumulation and movement of pesticide residues in soil, and the contamination of ground water by pesticides (Ryals et al. 1998; Kenna 1995; Klein 1990; Matthews et al. 1995).

NON_DESTRUCTIVE BIOMARKERS OF PESTICIDE EXPOSURE

For the biomonitoring study, non-destructive organismal and cellular **biomarkers** were used to quantify the possible effects of pesticide exposure. Measurements of **developmental stability** (e.g., fluctuating asymmetry), congenital birth defects (e.g., skeletal terata), genotoxicity (e.g., single and/or double strand breaks and/or alkali-labile sites, and micronuclei), clinical haematology (e.g., differential counts), general body condition (e.g., body mass-length relationships), and blood parasite load were assessed. These biomarkers were chosen because they can be used to assess the impact of potential pesticide exposure at the molecular, individual and potentially the population level. DNA

strand breaks, micronuclei formation and haematology endpoints are closely related to the episode of pesticide exposure, and are thus useful indictors of immediate effects of exposure. Biomarkers like body condition and parasite load (and birth defects) are useful predictors of individual organismal health in relation to chronic or ongoing pesticide exposure. These endpoints will return to normal levels once exposure is discontinued, but biomarkers like fluctuating asymmetry and skeletal birth defects are permanent, and can be used to indicate lasting effects of exposure that may lead to changes in individual and population survival. For instance, the presence of DNA strand breaks can be used to indicate exposure of an animal to a genotoxin at a point in time, but birth defects as a result of exposure will lead to lasting organismal effects (e.g., decreased fecundity, death) that are likely to impact the population. It is likely that the most effective way to determine what effect pesticide exposure can have on organisms is by using a multi-tiered approach where a number of biomarkers are used.

1. Developmental stability

Bilateral symmetry in the animal kingdom reflects a balance between two opposing forces, developmental noise and developmental stability. **Developmental noise** refers to a suite of processes that can lead to developmental instability; random differences in rates of cell division, growth and shape change, effects of thermal noise on enzymatic processes, and random differences in rates of physiological processes among cells (Palmer 1994). Developmental stability represents the ability to correct these errors and is conducted through negative feedback mechanisms that regulate enzyme activity within and among

cells, and central nervous and hormonal regulation of non-contiguous structures (Palmer 1994). It is hypothesized that under stressful conditions, organisms will allocate energy to more important life history traits such as growth and reproduction, or to fighting stress itself, rather than to maintaining developmental stability (Mitton 1993). This re-direction of energy will favour developmental noise and the developmental trajectory will slowly move away from bilateral symmetry, resulting in an asymmetrical phenotype.

A commonly used measure of developmental stability is fluctuating asymmetry (FA). FA is defined as random deviations from perfect bilateral symmetry with no genetic predisposition towards one side or the other being larger (Palmer and Strobeck 1986; Palmer and Strobeck 1992; Parsons 1992; Møller and Swaddle 1997). FA can be measured at the individual level (referred to as subtle asymmetry by Palmer 1994) and is generally measured as absolute right minus left values of meristic or metrical traits, or at the population level, by comparing the variance of FA distributions between populations (Gangestad and Thornhill 1998). Considering that stress reduces the potential for correcting developmental errors, or by increasing noise itself (Palmer 1994), the more stressed an individual, the more likely it is to exhibit higher FA than unstressed organisms (Clarke 1993; Møller 1993). FA is reputed to be a useful general biomarker of environmental stress. It has been shown to respond to a wide range of stresses (e.g. noise, population density, environmental disturbance, exposure to pesticides and metals) and has been correlated with many life history traits (e.g., mait selection, parasite resistance, survival) (see Møller and Swaddle (1997) and Polak (2003) for a review).

An attractive feature of using FA as a biomarker is its relative ease in measurement. As long as researchers have access to callipers and bilaterally paired traits, FA data can

easily be generated. FA has been measured on traits ranging from teeth to feathers to leaves and in organisms ranging from plants to humans (Møller and Swaddle 1997). Even if data generation is straightforward, the analysis of FA is not. Interpretation of patterns and the statistical testing of data can often be the most problematic component of a study dealing with FA (Knopper and Mineau 2002). Because of these problems, the use of FA as a biomarker has generated both staunch advocates as well as harsh critics (Leung, Knopper and Mineau 2003). Advocates have stated that "...morphological variability [FA] may provide a valuable early indicator of environmental and genetic stress" (Leary and Allendorf 1989), and that "...no existing techniques designed to detect possible adverse impact on native animal populations can match the FA approach for sensitivity" (Clarke 1993), and that "...FA is known to be diagnostic for a wide range of environmental and genetic stressors... Indeed, it may be the best measure we have" (Polak and Trivers 1994). Conversely, other researchers have been less enthusiastic regarding the potential of FA as a biomarker and have said that "...FA may be a poor general predictor of either stress or fitness" (Leung and Forbes 1996), and "...just how powerful is this tool [FA], and how reliable? Perhaps very, but those wishing to apply it must recognize that the biological signal is exceedingly small and that not all deviations from symmetry provide a useful signal" (Palmer 1996). Due to this reported inconsistency, the utility of FA as a biomarker is questionable. Further studies need to be conducted in order to validate its use under differing situations.

2. Developmental Toxicology

Birth defects can be caused by exposure of the conceptus to anthropogenic chemicals (e.g., pesticides, pharmacological agents) capable of genetic and non-genetic damage. During the preimplantation phase, embryo organogenesis, and fetal and neonatal development, anthropogenic toxicants can lead to excessive cell death, cell proliferation, and inhibited cell-cell communication, all of which can lead to congenital birth defects (Manson et al. 1982; Rogers and Kavlock 1996). Recent wildlife biomonitoring and human epidemiological studies have revealed a relationship between animals and humans living in areas of pesticide use and teratogenic outcomes. For example, Ouellet et al. (1997) observed high rates of hindlimb deformities in wild green frogs (*Rana clamitans*), northern leopard frogs (*Rana pipiens*), American toads (*Bufo americanus*), and bullfrogs (*Rana catesbeiana*) living in agricultural sites exposed to pesticide runoff; Linzey et al. (2003) reported a correlation between soil, water and body fat concentrations of DDT, DDE and PCB in marine toads (*Bufo marinus*) and whistling frogs (*Eleutherodactylus johnstonei*) in Bermuda, with limb deformities in subadult, adult and newly-metamorphosed animals; and Bell et al. (2001) suggested that exposure to several classes of pesticides (e.g., organophosphates, pyrethroids, carbamates, and endocrine disruptors) during the 3rd to 8th week of pregnancy in humans was associated with fetal death due to congenital anomalies. Moreover, records from a wildlife sanctuary in central Ontario have revealed that an increasing number of small mammals found living on golf courses have required rehabilitation because of retinal damage and blindness (unpublished data, Aspen Valley Wildlife Sanctuary, Rosseau Ontario).

3. Genotoxicity

Normal wear and tear resulting from cellular processes continuously produces transient DNA that is in a state of damage. For instance, strand breaks, the result of broken phosphodiester bonds by oxygen radicals, and abasic sites, the loss or alteration of purine or pyrimidine bases as a result of destabilization of the N-glycosyl bond between the base and the deoxyribose backbone, are common natural occurrences (Ruddon 1990; Shugart 1992). In fact, no less than 200 oxidative reactions occur daily in human cells (Halliwell 2000). Maintaining DNA integrity is of paramount importance to all living things and for this reason organisms possess mechanisms for the repair and synthesis of their genetic material (e.g., general and specific excision-repair pathways). Physiological stresses can lead to dysfunction of these mechanisms and when coupled with chemical toxicity that can lead to an increase in DNA damage, the result is above normal levels of damaged genetic material (Shugart et al. 1992). Considering that DNA damage is generally the first step in the cascading events leading to cancer and eventual cellular and organismal death (and the possible heritable transfer of mutation and infertility), the detection of genetic damage is essential for biomonitoring studies.

Genotoxicty tests are based on either detecting DNA damage or the biological effects of DNA damage (Tice et al. 2000). Tests of genotoxicity range in duration, expense, involvement, cell type and vary depending on the organism used. The two genotoxicity tests used in this thesis, the **comet assay** and the erythrocyte **micronucleus assay**, can be conducted non-lethally with relative ease, with very small amounts of biological sample, have low running costs, and quantifiable data can be rapidly generated. DNA strand breaks can be repaired quite rapidly so the positive results from the comet assay can be used to

indicate recent exposure of voles to a genotoxic compound. Micronuclei form as a result of genotoxic damage in erythrocyte stem cells (see below). In the micronucleus assay, micronuclei are visualized in immature red blood cells (polychromatic erythrocytes, PCE), and since the life span of red blood cells is approximately 120 days (Israels 2002), positive results from the micronucleus assay can be used to indicate chronic exposure to genotoxic compounds. Both of these assays have recently been employed in the assessment of genetic damage in white stork (*Ciconia ciconia*) exposed to heavy metals (Pastor et al. 2001), in wild rodents (*Ctenomys torquatus*) living in coal mining regions (Silva et al. 2000) and areas of high vehicular traffic (Heuser et al. 2002), in tadpoles living in agricultural and industrial sites (Ralph and Petras 1996), and in brown trout (*Salmo trutta*) living in contaminated rivers (Sánchez-Galán et al. 1998).

3.1 The Alkaline Comet Assay

The comet assay, also known as the single-cell gel electrophoresis test, is a method of detecting strand breakage in virtually any nucleated cell and can be used as a biomonitoring tool. The overall technique for conducting the comet assay is common to all researchers but the two most important steps, 1) ensuring the proper pH for appropriate solutions, and 2) finding the most effective voltage and milliamperage for electrophoresis, are specific to cell type, laboratory, and the questions being researched. Under neutral pH conditions, only double stranded DNA breaks can be revealed using the comet assay. Under alkaline pH (> 13) conditions, double and single strand breaks as well as alkali labile sites (expressed as single strand breaks) can be detected. Considering that chemical genotoxins cause orders of magnitude less double strand breaks than single strand breaks

compared to radiation exposure (Tice et al. 2000), the alkaline comet assay is a more useful tool for biomonitoring than the neutral version. If the voltage used to electrophoresis cells is too low, generally less than 1V/cm between the anode and cathode, DNA will not migrate. However, if voltage is too high, undamaged cells will be pulled apart and will exhibit the comet shape associated with damage. Thus, conditions need to be optimized so that only true damage can be detected.

The general technique starts with putting nucleated cells of interest into a single cell suspension mixed with melted agarose and casting the mixture onto frosted glass slides (Tice et al. 2000; Krause et al. 2001) or into individual plastic chambers affixed to a piece of GelBond acetate film (McNamme et al. 2000). Once the agarose has solidified, gels are placed in a lysing solution, comprised of salts and detergents (Tice et al. 2000). From the lysis buffer, gels are placed in the alkaline electrophoresis solution and DNA is allowed to unwind. This step is followed by electrophoresis. From here, gels are placed in a neutralizing solution in order to fix the migrated DNA. Gels are then dehydrated in 100% ethanol so they can be stored indefinitely and scored at a later date.

To quantify DNA damage, gels are stained with a DNA stain and the cells scored for DNA migration under a fluorescent microscope. Damaged cells appear like astronomical comets, with long tails of DNA migrating from the center of the exposed nuclceoid (Figure 1). Damage is generally quantified using two main values: **tail length** and **tail moment** (i.e., tail length x %DNA in the tail), a measure of DNA migration (Collins et al. 1997; Tice et al. 2000). The most informative metric is currently under debate by the comet assay community, mainly because computerized imaging programs tend to compute these metrics differently.

3.2. Micronucelus Assay

The micronucleus assay is a common toxicological test used to measure chromosomal damage in relation to genotoxic damage at the level of the stem cell (Hayashi et al. 1994; EPA 1998; Krishna and Hayashi 2000). Micronuclei are whole chromosomes, or pieces of chromosomes, not incorporated into the nucleus during cell division and left behind in the cytoplasm. The process of erythropoiesis (maturation of erythrocytes) has been used as the vehicle for *in vivo* measurements of micronuclei (Hayashi et al. 1994; Krishna and Hayashi 2000). Genotoxic insult on stem cells can cause the formation of micronuclei in the proerythrocyte through direct clastogeic action (causing breaks of the chromosome) or by acting on the molecules needed for mitosis (e.g., spindle dysfunction). As proerythrocytes continue to divide they mature into polychromatic erythrocytes (PCE), also known as reticulocytes, young red blood cells containing RNA (Israels 2002). Just prior to PCE formation, the main nucleus is ejected, so visualization of MN in PCE can be conducted.

An increase in the number MN-PCE over that found in control animals is an indication of genotoxic damage (Hayashi et al. 1994; EPA 1998; Krishna and Hayashi 2000). For manual scoring of MN-PCE, blood smears are made and stained with a DNA binding dye. The number of MN-PCE out of 2000 PCE is accepted as the standard for obtaining an unbiased estimate of MN-PCE levels in the exposed animal (Krishna and Hayashi 2000). The number of PCE in a known number of total erythrocytes (generally

1000) can be used as an indication of cytotoxicity and rate of erythropoiesis (Hayashi et al. 1994).

4. Clinical Haematology and Body Condition

Leukocyte differential counts are a standard diagnostic test to quantify changes in leukocyte levels and are the cornerstone in laboratory hematology for yielding clinically useful information about organismal health and disease (Tvedten et al. 1988; Houwen 2001). The differential count provides a pathology report on the morphological appearance of blood cells and the frequency in which they appear (Duncan et al. 1994; Houwen 2001). In general, elevated levels of granulocytes (i.e., neutrophils, eosinophils, basophils) and monocytes are indicative of acute infection, malignant tumors and leukemia, and organismal stress (Smith 1996). Elevated eosinophils are the hallmark of multicellular parasitic disease and elevated monocyte counts may be seen in animals that are acutely stressed and experiencing hormonal abnormalities (Smith 1996). Depressed granulocyte levels are the most common manifestation of chemically induced bone marrow damage (Smith 1996). Elevated levels of circulating lymphocytes (e.g., T-cells and B-cells) can be attributed to certain types of cancer and parasitic or bacterial infection (Taussig 1984).

Body condition of an animal refers to its energetic state (e.g., fats stores) and it is assumed that animals in good condition have higher energy reserves than those individuals in poorer condition (Schulte-Hostedde et al. 2001). Rather than directly measuring fat reserves, which necessitates euthanizing the animal, an individuals' condition can be non-lethally quantified using indices based on the relationship between body mass and body length (Krebs and Singleton 1993; Schulte-Hostedde et al. 2001; Blackwell 2002). Indices

of body condition are commonly based on the residuals of a linear regression of body mass on body length (e.g., tip of nose to base of tail). With this index, individuals with positive residuals are considered to be in better condition than predicted for their size, with the opposite true for individuals with negative residuals (Krebs and Singleton 1993; Schulte-Hostedde et al. 2001; Blackwell 2002). Chronic exposure to pesticides and other anthropogenic toxicants has been associated with health concerns like cancer, **immunosuppression**, and increased susceptibility to infection (e.g., increased blood parasite load), all of which could negatively affect the body condition of an organism.

MEADOW VOLES AS AN INDICATOR SPECIES

The use of small mammals as indicator species in biomonitoring studies is well documented. Choosing a suitable species for a study depends on the food habits of the animal, their abundance, and availability, and of course, the compound being assessed (Talmage and Walton 1991). In their review paper that evaluated the use of small mammals as monitors of environmental contaminants, Talmage and Walton (1991) suggest that herbivorous mammals are well suited to studies dealing with contaminants that are deposited directly on vegetation. On golf courses, pesticides are applied directly to the turf and can disperse in the air to adjacent vegetation as pesticides are applied.

Meadow voles (*Microtus pennsylvanicus*) are ideal candidates to measure the effects of pesticide exposure because they are likely to come into contact with pesticides on golf courses through many routes. Voles may absorb pesticides through the pads of their feet after walking on treated turf, orally after ingesting treated items, or from inhalation of

pesticide drift after application. Meadow voles also have relatively small home ranges (males: 400m^2, females: 200m^2 (Ambrose III 1969; Douglass 1976; Madison 1980; Ostfeld et al., 1983) and will likely spend their entire life on the course in which they are found. Therefore, it can be expected that any pesticide-related effects observed in these animals would be the result of contact with pesticides used on their home golf course. A detractor from using meadow voles as indicator species is that they have a relatively short life span (3-5 months in the wild), so chronic diseases like cancer that take time to develop may never be observed in these animals. Further, vole populations undergo as of yet, unpredictable population cycles (Getz 1960; Getz 2001), so it is possible that sample sizes may widely vary from year to year and from site to site.

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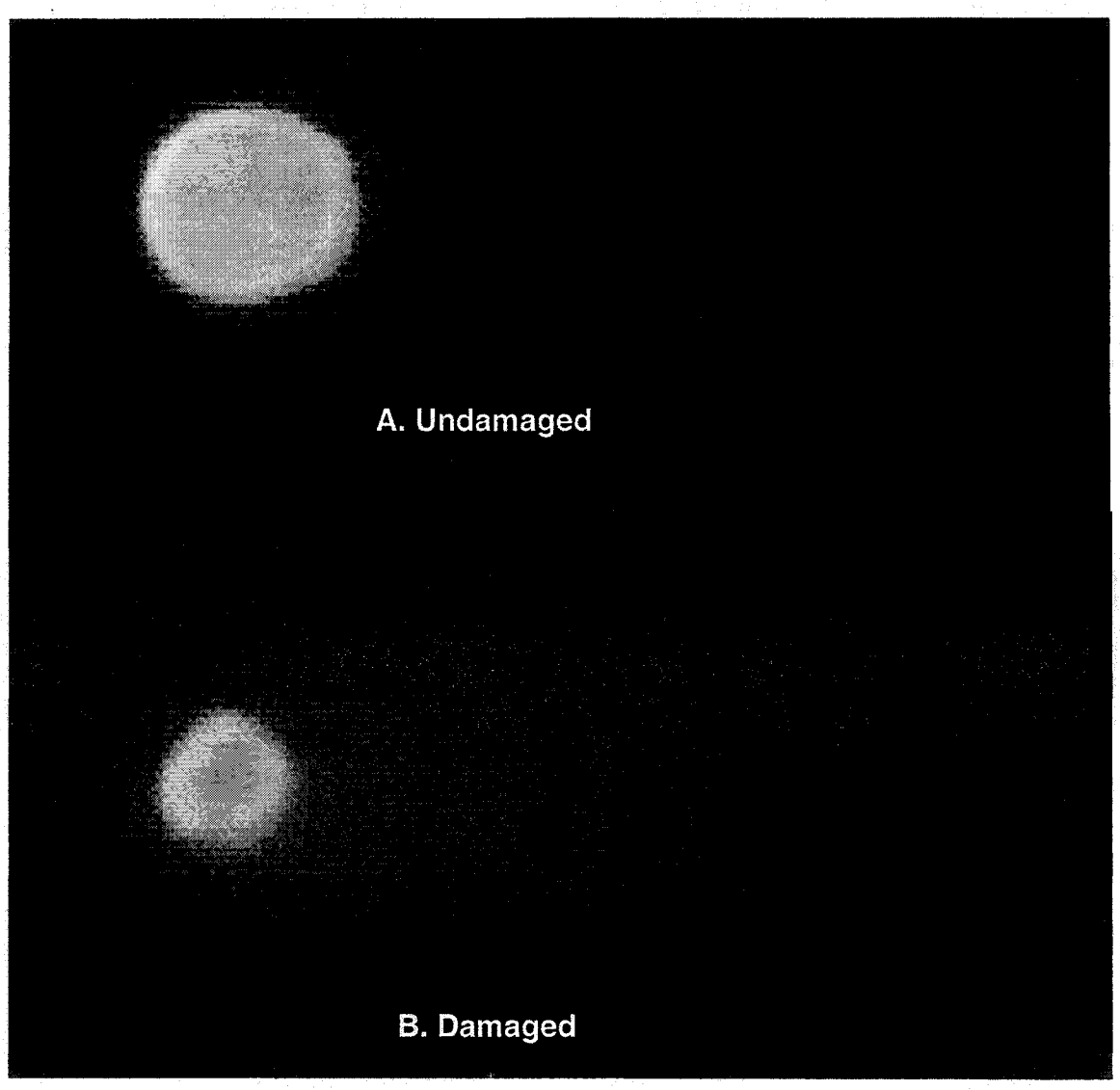
Figure 1.: Image of an alkaline comet taken with a 40X oil immersion objective lens (total magnification 330X) stained with SYBR Gold stain. A) Undamaged cell, B) damaged cell.



CHAPTER 2:

CARCINOGENIC AND GENOTOXIC POTENTIAL OF COMMONLY USED TURF PESTICIDES ON GOLF COURSES

INTRODUCTION

Strong emotional, financial and scientific interest surrounds studies dealing with the effects of pesticide exposure on human, animal, and environmental health. One of the dominant health effects of concern in relation to pesticide exposure is the occurrence of cancer (Zahm and Blair 1992; Dich et al. 1997; Standing Committee on Environment and Sustainable Development, 2000), and studies making an effort to correlate the two can be especially controversial. While some scientific studies have suggested relatively strong associations between pesticide exposure and cancer, others have reported no such relationships (see below). As a result of this ambiguity, and due to the direct alarm of their constituents, many municipal governments throughout Canada are implementing bylaws, or are in the process of drafting them, that regulate the public and private use of pesticides (e.g., Province of Ontario: Waterloo, Stratford, Dundas, Ottawa-Carleton; Province of Quebec: Lachine, Dorval, Entrelacs, Hudson, Chelsea; Province of Nova Scotia: Halifax). The objectives and specific details of these bylaws vary between municipalities (e.g., areas and situations of allowable application, times that provisions come into effect, etc.) but the underlying theme in each appears similar; bylaws are in place to regulate the public and private use of pesticides to protect the health of citizens and the environment (114957 Canada Ltée (Spraytech, Société d'arrosage) *v.* Hudson (Town) 2001 SCC 40)). Opponents of these bylaws argue that there is a lack of scientific evidence linking pesticide exposure to poor human and environmental health (Meech and English 2000) and assert that municipalities should not have the authority to ban pesticides already approved at the federal level. Despite these objections, municipal

governments and the Supreme Court of Canada, which ruled nationally in 2001 on the authority of such bylaws (114957 Canada Ltée (Spraytech, Société d'arrosage) *v.* Hudson (Town) 2001 SCC 40), have based their decisions in part on the **precautionary principle**, stating that "...lack of full scientific certainty should not be used as a reason for postponing measures to prevent environmental degradation..." (paragraphs 30-32, 114957 Canada Ltée (Spraytech, Société d'arrosage) *v.* Hudson (Town) 2001 SCC 40). Following the Supreme Court's ruling, more municipalities are initiating cosmetic pesticide use phase-outs, and as of September 2002, the Province of Quebec has banned the use of pesticides on all provincially owned areas and intends to phase out residential lawn pesticide use by 2005 (Pesticide Management Code, Province of Quebec, 2002).

In many municipalities, the use of pesticides on golf courses also falls under the newly created bylaws. A wide range of pesticide products is available to golf course superintendents to help maintain attractive and healthy turf. With over 1600 golf courses in Canada (personal communication Royal Canadian Golf Association) and between 400 and 600 new courses created each year in Canada and the U.S.A., there appears to be increasing potential for unintentional human and animal exposure to pesticides. I am unaware of any ongoing studies on health outcomes of animals or people likely to come into contact with golf course pesticides. In light of the debate around pesticide exposure and the onset of cancer that has lead to controversial Canadian municipal bylaws regulating pesticide use, it seemed timely to review some of the debated epidemiological research that deals with the relationship between pesticide exposure and cancer, and to review and update the literature on the in vivo and in vitro mammalian **carcinogenic** and genotoxic potential of these pesticides. It is my intention to unite information from various sources so those interested

specifically in the carcinogenicity and genotoxicity of pesticides commonly used on golf courses can refer to one comprehensive and updated resource.

EPIDEMIOLOGICAL STUDIES

Epidemiological studies fall into two main categories: **case-control** and **cohort studies**. Case-control studies are studies in which individuals that already exhibit a certain condition (e.g., cancer) are compared with individuals who do not, and researchers try to identify differences (e.g., pesticide exposure) between the two groups that may explain the condition. Cohort studies are those where groups of individuals with known differences (e.g., one cohort has been exposed to pesticides and the other has not) are followed over time and the occurrence of certain conditions (e.g., cancer) compared. While some epidemiological studies suggest convincing relationships between cancer and exposure to pesticides, other studies suggest just the opposite. The following case-control and cohort studies are not meant to act as a comprehensive review of the epidemiological literature. Rather they have been included in this review because they focus on the relationship between pesticides common to golf courses and cancer, and because they illustrate both sides of the pesticide exposure-onset of cancer debate. For quick reference, Table 1 cites these studies and indicates their results.

a) Case-Control Studies

In their review paper, Zahm and Blair (1992) cite eight case-control and cohort studies from around the world that have reported increased incidence of **non-Hodgkin's lymphoma** (NHL) in relation to pesticide exposure. Hardell et al. (1981) reported that people in Sweden who were exposed to phenoxyacetic acids and chlorophenols had a 6-fold greater risk of developing NHL and **Hodgkin's disease** than those individuals who were not exposed. Zahm et al. (1990) reported that farmers in Kansas who used phenoxy herbicides had a 2-fold higher risk of developing NHL than farmers who did not. For those farmers using phenoxy herbicides more than 21 days a year, risk of NHL increased 7-fold. Waddell et al. (2001) combined the results from three population-based case-control studies where farmers, non-farmers, or their respective respondents were interviewed to determine a relationship between cancer and pesticide exposure. Among direct respondents to the interview, the relative risk of small lymphocytic lymphoma was associated with the use of diazinon (**odds ratio**: (OR)= 2.8), fonofos (OR=2.6), phorate (OR=2.3), terbufos (OR=2.2), and malathion (OR=1.9). It needs to be noted that even though Waddell et al. (2001) found an association between small lymphatic lymphoma and organophosphate use, they did not find significantly elevated rates of NHL in farmers compared to non-farmers. McDuffie et al. (2001) conducted a population-based case-control study among men from 6 Canadian provinces to determine the relationship between lifetime exposure to pesticides and NHL. Odds ratios were based on exposure to pesticide classes (e.g., herbicides: phenoxyherbicides vs. thiocarbamates vs.dicamba containing classes), and to individual compounds within these classes (e.g., herbicides: 2,4-dichlorophenoxyacetic acid (2,4-D), glyphosate, etc.; insecticides: carbaryl, dichloro-

diphenyl-trichloroethane (DDT), etc.; fungicides: captan, mercury containing fungicides, etc.). Odds ratios were adjusted for statistically significant medical variables like previous cancers, familial cancers, measles, mumps and allergy desensitization. In their final analysis, McDuffie et al. (2001) found that the risk of NHL was significantly increased (OR>1) by exposure to phenoxyherbicides and dicamba, to carbamate and organophosphorous insecticides, and amide fungicides. Among individual compounds, risk of NHL was significantly increased by exposure to the herbicides 2,4-D, mecoprop and dicamba, to the insecticides malathion, DDT, carbaryl, aldrin and lindane, and to the fungicide captan, and other sulfur containing fungicidal compounds. When McDuffie et al. (2001) revised their analyses and calculated ORs using multivariate models that also included information about personal cancer history and the history of cancer in first-degree relatives, risk of NHL was only significantly related to exposure to dicamba-containing herbicides (OR=1.96), to mecoprop (OR=2.22), and to aldrin (OR=3.42).

b) Cohort Studies

Lynge (1998) conducted a cohort study in Denmark and investigated the incidence of cancer in 2119 people from 1947 to 1993. These subjects worked in factories that produced phenoxy herbicides from 1947 to 1951, a time when standards of industrial hygiene were likely lower than they are today. Lynge (1998) suggested that workers had a marginally lower incidence of cancer than the rest of the Danish population. Of the 2119 individuals, 204 individuals were found to have cancer, whereas the expected

incidence was 234. Cancer of the stomach, colon, rectum, pancreas, lung, breast, prostrate, cervix, testes, kidneys, bladder, skin, blood and esophagus, were not significantly elevated compared to the general population. Of the 204 cases of diagnosed cancer, soft-tissue **sarcoma** (STS) and NHL were slightly elevated above expected. The occurrence of STS was observed 4 times when it was expected 2.5 times, and NHL was observed 6 times when it was expected 5 times. However, only STS, but not NHL, was significantly elevated in the cohort. Zahm (1997) conducted a cohort-study involving over 32, 000 lawn care company employees in order to identify relative risk of mortality due to occupational exposure to pesticides. Results indicated that the cohort had significantly lower mortality rates due to cancer (45 of 32,600 people) than expected (60 of 32,600 people) based on mortality rates of the general U.S. population (Zahm, 1997). Limitations to this study included the relatively young age and health status of the cohort, limited exposure to pesticides because of short employment time at the lawn care company, and brief follow-up on the health of individuals. In contrast, results from a historical cohort mortality study conducted on farmers from Saskatchewan, Canada, indicated that death associated with NHL was significantly related to herbicide use (Wigle et al. 1990). Pesticide exposure data were taken from a 1971 Agricultural Census and merged with mortality records from a Census of Population. It should be noted that the majority of herbicides used by these farmers were 2,4-D formulations. Farmers that applied herbicides on 100 acres or fewer had a risk ratio (RR) of 1.3, while farmers that worked 100-249 acres had a RR of 1.9 and farmers with 250 acres or more had a RR of 2.2. Kross et al. (1996), at the request of the Golf Course Superintendents Association of America, conducted a proportionate mortality cohort study using death records from 686

golf course superintendents, and compared their rates and reasons for mortality to the general U.S. Caucasian-male population. While Kross et al. (1996) did not identify specific risk factors due to the variety of potential chemical exposures related to mortality because of the difficulty in determining the intensity and duration of historical exposures, two distinct patterns did emerge from the study. Death due to smoking-related diseases and death due to cancers commonly associated with chronic pesticide exposure, were elevated in the cohort compared to the reference population. **Proportionate mortality ratios** (PMR) associated with smoking-related diseases like lung and esophageal cancer and arteriosclerotic heart disease were 234, 139 and 140 respectively. Proportionate mortality ratios associated with pesticide related diseases like brain cancer and NHL were 234 and 237, respectively.

GOLF COURSE USE OF PESTICIDES

Up to the twentieth century, golf course superintendents employed weed pickers to remove unwanted plants from golf courses, used unprocessed manure as turf fertilizer, sprayed nicotine as an insecticide, and utilized grazing cattle, sheep and rabbits to maintain a well clipped and healthy turf (Beard 1989). As mentioned in Chapter 1, superintendents today rely on more sophisticated equipment and have many more pesticide products available to help maintain a well-groomed and healthy turf. As part of the biomonitoring component of this thesis, a survey of pesticide use from the participating golf courses was conducted. Superintendents from each of the courses provided a copy of their pesticide use records for 1999 to 2002. The products used were generally the same from course to

course, and even though the amount of product applied (e.g., kg/ha) varied between courses, the use ranking of products was similar. Fungicides with the active ingredients iprodione, chlorothalonil, and phenyl mercuric acetate (PMA) were predominately used (but PMA was not used after 2001 due to a national phase out), followed by herbicides containing 2,4-dichlorophenoxyacetic acid (2,4-D) and glyphosate, and insecticides containing diazinon.

The cancer-causing potential of these active ingredients is under debate. Pesticides that are genotoxic can trigger the onset of cancer by directly causing DNA damage or they can be non-genotoxic (i.e., **epigenetic**) and capable of initiating any number of the steps in the onset of cancer without actually causing genetic damage (Ratitsky et al. 2000). The following text describes the chronic effects of exposure to these active ingredients in the laboratory and some of the mammalian in vitro and in vivo data available for each compound. The 1986 U.S. EPA carcinogenicity ratings are given for each compound. The main factor upon which these ratings are based was in vivo tumor findings in animals and humans (U.S. EPA 1996), with very little consideration given to epidemiological evidence. Thus, a 1986 rating may be contrary to recent epidemiological or laboratory evidence. Presently the EPA is in the process of revising its carcinogenicity ratings (U.S. EPA 1996). These new guidelines are expected to focus more on a weight-of-the-evidence approach, taking all data into consideration rather than weighing heavily on lab-based tumor studies (U.S. EPA 1996). For quick reference, Table 2 cites *in vivo* and *in vitro* mammalian studies indicating either positive or negative genotoxic potential for each active ingredient, and the genotoxic assays used to determine that potential. In some cases many studies have been

conducted on each product and for brevity these studies have not been described in the text but simply cited in the tables.

1. Fungicides

a) Iprodione: Iprodione is the active ingredient in products such as Kidan® and Rovral Green®. It is a dicarboximide contact fungicide that has protective and curative actions and is used to control brown patch, dollar spot, and many types of blight (Beard 1989; Tomlin 1997). Iprodione inhibits the germination of fungal spores and growth of mycelium by inducing lipid peroxidation via oxygen activation (Radice et al. 1998). The environmental fate of iprodione varies depending on soil type, soil acidity and clay content. Half-life in soil ranges from 7 to over 60 days, with an average half-life of 14 days (PIP Iprodione 1996). In water iprodione breaks down rapidly in both aerobic and anaerobic conditions (PIP Iprodione 1996).

Iprodione does not appear to exert genotoxic effects (Tomlin 1997; PIP Iprodione 1996; U.S. EPA 1998). With in vivo studies, iprodione produced no chromosomal aberrations, sister chromatid exchange or forward mutation in Chinese hamster ovary cells (CHO), and did not induce the formation of micronuclei (U.S. EPA 2001). However, iprodione does appear to have epigenetic effects that lead to cancer. Proprietary studies by Aventis Crop Science indicated that inhibition of steroid and androgen biosynthesis by iprodione and its metabolites produced Leydig cell tumors in rats and hepatic cancer in male and female mice via a non-linear dose response relationship (U.S. EPA 2001). The

U.S. EPA has classified iprodione as a Group B2 compound (i.e., probable human carcinogen with sufficient data on animals and inadequate or non-data for humans).

b) Chlorothalonil: Chlorothalonil is a broad spectrum, non-systemic foliar fungicide and is the active ingredient in products such as Daconil®, Echo®, and Bravo®. Its mode of action is to conjugate with, and thus deplete thiols (particularly glutathione) from germinating fungal cells (Tomlin 1997). The result is a disruption of glycolysis and eventual cell death. Increased soil moisture and ambient temperatures increase the breakdown of chlorothalonil in soil and its half-life ranges between 1 and 3 months (PIP Chlorothalonil 1996). Chlorothalonil does not appear to be taken up by plants and may remain on plant surfaces for some time after application (Tomlin 1997).

Chronic exposure to chlorothalonil is known to produce severe eye and skin irritation, is cytogenic and carcinogenic. Chronic dietary treatment of chlorothalonil (175 mg/kg/day) to rats and mice resulted in benign and malignant tumors of the forestomach and kidneys, and hyperplasia, a process that can lead to a cancerous tumor, in the renal proximal tubules (Wilkinson and Killeen 1996). Chlorothalonil carcinogenicity appears to be related to epigenetic effects rather than through genotoxic mechanisms (Wilkinson and Killeen, 1996; Rosenkranz 1999; Rakitsky et al. 2000). Forestomach and kidney cancers can arise through non-genotoxic mechanisms such as cytotoxicity, cell necrosis, cell proliferation and hyperplasia that are initiated by toxicant exposure (Wilkinson and Killeen 1996). Chlorothalonil is classified by the International Agency for Research on Cancer (IARC) and the U.S. EPA as a possible human carcinogen (IARC Group 2B (IARC, 1999), U.S. EPA Group C (U.S. EPA, 1999)).

In some cases chlorothalonil has been shown to have genotoxic properties, but generally only at low doses and only during initial exposure. At higher doses and extended exposure periods, cytotoxic effects quickly over-shadow genotoxic effects. Two studies in particular, Lebailly et al. (1997) and Godard et al. (1999), have shown chlorothalonil to be genotoxic by use of the comet assay under short exposure times, but quickly observed enhanced cytotoxicity leading to highly damaged cells indicative of apoptosis with increasing concentration and time. Lebailly et al. (1997) showed greater in vitro DNA damage (e.g., single and/or double strand breaks and/or alkali-labile sites) in human lymphocytes exposed to increasing concentrations of chlorothalonil (10, 50, 100 and 500μM). After an hour of exposure cell viability remained unchanged at all dose concentrations but increasing DNA damage occurred in a concentration-dependent manner with damage above control values detected at 50μM and up. After 24 hours of exposure, significant differences between concentration and control cells were observed at the 10μM level, but this came with a concomitant decrease in cell viability associated with a large proportion of highly damaged cells (i.e., apoptotic cells). Godard et al. (1999) measured chlorothalonil-related in vitro DNA damage in CHO cells and in vivo damage in various tissues of male Sprague-Dawley rats. The in vitro study resulted in the dose-related appearance of DNA strand breaks. In vivo studies failed to detect any DNA damage, regardless of dose (200 or 2000 mg/kg p.o. in corn oil) or time of exposure (2, 6, or 24 hrs), presumably due to metabolic and mechanistic aspects. Similar to Lebailly et al. (1997), cytotoxicity increased in relation to dose as indicated by increasing numbers of highly damaged cells, but unlike Lebailly et al., it was observed after 1 hour of exposure and

mainly at higher doses. For a review of studies dealing with the genotoxic potential of chlorothalonil prior to 1993, refer to Dearfield et al. (1993).

c) Phenylmercury acetate: Phenylmercury acetate (PMA), an organic form of mercury, also known as phenylmercuric acetate, is the active ingredient and common name for a fungicide with eradicant action to control snow molds and *Fusarium sp.* (Tomlin 1997). The vast majority of available toxicity studies for organic mercury compounds are for methylmercury. It is likely that the mode of action of PMA is similar to that of other organic mercurial compounds but this mode is not fully understood (Hassett-Sipple et al. 1997). What is known is that organic mercury (e.g. methylmercury) is readily absorbed (up to 95%) through the gastrointestinal tract and, to a lesser extent (3-5%) through the skin (Hassett-Sipple et al. 1997).

The effects of organic mercury have been measured in organisms from bacteria to humans and the results are complex (De Flora et al. 1994). Generally, mercurial compounds appear to produce clastogenic effects (i.e., chromosomal breaks) in eukaryotes by binding sulfhydryl groups and inhibiting mitotic spindles. Whether PMA acts through this mechanism has yet to be determined. The U.S. EPA considers all organic mercury products as Group C possible human carcinogens (Hassett-Sipple et al. 1997) and chronic exposure to PMA induces genotoxicity and teratogenicity. Genotoxic effects of PMA have been reported by Lee et al. (1997) who showed that concentrations of PMA ranging from 1 to 30 μM mixed with human lymphocytes in vitro produced significant increases in sister chromatid exchange and endoreduplication of chromosomes (i.e., replication of chromosomes without subsequent cell division) in a concentration dependent manner. As

of December 31, 1995, PMA was discontinued for use in Canada as a turf pesticide, with a complete phase out as of January 1, 2001.

2. Herbicides

a) 2,4-Dichlorophenoxyacetic acid: Commonly known as 2,4-D, this systemic herbicide is selectively toxic to broad-leaved plants, and is the primary active ingredient in the products Killex® and Par III®. 2,4-D is absorbed through foliage and is translocated to the roots of the plant where it acts as an auxin mimic and resultant growth disrupter (Tomlin 1997). Microbes quickly break down 2,4-D in soil and its resultant half-life is generally less than one week (PIP 2,4-D 1996). It can persist for several weeks or more in water (PIP 2,4-D, 1996).

Most laboratory studies seem to indicate that 2,4-D has no genotoxic properties but its epigenetic effects have been documented. Gollapudi et al. (1999) reported that 2,4-D did not produce chromosomal aberrations or forward gene mutations at concentrations as high as 1400ug/ml when added to rat lymphocytes in vitro. Charles et al (1999a) reported that 2,4-D did not induce increased micronuclei in mouse bone marrow cells in vivo, and Charles et al. (1999b) reported that 2,4-D and 7 of its salts did not produce reverse mutations in *Salmonella* or unscheduled DNA synthesis in rat hepatocytes in vitro (concentrations range from 0.5 to 478 ug/ml) or in vivo (either 400 or 1000 mg/kg given by gavage). Figgs et al. (2000) measured the relationship between micronuclei frequency and blood lymphocyte proliferation (e.g., replicative index: RI) and urinary 2, 4-D levels in pesticide applicators who exclusively sprayed 2, 4-D for 3 months. While micronuclei

frequencies, the genotoxicity biomarker, did not differ between applicators and non-applicators, RI, the epigenetic biomarker, did. RI was significantly higher in applicators compared to non-applicators, independent of alcohol and tobacco use. Holland et al. (2002) also found non-genotoxic but epigenetic effects of 2,4-D in vitro and in vivo using human lymphocytes.

Tremendous debate has followed epidemiological studies that have investigated the relationship between 2,4-D and cancer in animals and humans. Hayes et al. (1991) conducted a hospital-based case-control study to investigate the relationship between canine malignant lymphoma (CML), cancer with similar etiology and histology as human NHL, and chemical exposure, particularly 2,4-D. The results of this study indicated that dogs whose owners used 2,4-D or who hired professional lawn care companies to treat grass, appeared to have CML 1.3 times more often than dogs whose owners used no pesticides. Moreover, the relative risk of CML in dogs appeared to increase 2-fold with 4 or more yearly applications of 2,4-D. In 1992, The Industry Task Force II on 2,4-D Research Data sponsored a review (Carlo et al., 1992) to critically evaluate the findings and methodologies of the Hayes et al. (1991) study. Specific concerns were based on the definition of exposure and whether animals were exposed to pesticides other than 2,4-D, pooling of animals in a control group, method of response, and ambiguities regarding exposure classification. Hayes et al. (1995) responded to these critiques with a detailed reanalysis of their 1991 data using a more stringent definition of 2,4-D exposure. The conclusions of the 1995 study were essentially the same as the original. Hayes et al. (1995) did acknowledge, however, that the magnitude of the relationship between 2,4-D exposure and CML was small (OR 1.3-1.4) and that their study did not prove causation between CML and 2,4-D,

but suggested that data did highlight concerns with the use of such chemicals. Another review of the Hayes et al. (1991 and 1995) study was conducted by Kanenee and Miller (1999), again sponsored and funded by The Industry Task Force II on 2,4-D Research Data. This critique, however, did not raise any new points beyond those that had already been addressed by Hayes et al. (1995).

In 1989 a panel of 13 scientists was convened to review the 2,4-D literature published in the fields of toxicology and epidemiology, in order to make a weight-of-the-evidence judgment about the carcinogenicity of 2,4-D (Ibrahim et al., 1991). In 1994, the U.S. EPA conducted a very similar weight-of-the-evidence-study to evaluate the association between 2,4-D and NHL (U.S. EPA, 1994). The general findings of both panels was that laboratory studies conclude that 2,4-D has minimal or equivocal carcinogenic potential, that cohort-epidemiology studies suggested the same, but case-control studies usually indicate an association between NHL (but not other cancers) and 2,4-D or other phenoxy herbicides. It should be noted that the Ibrahim et al. (1991) study took place before recent laboratory and epidemiological studies which showed positive associations between exposure to 2,4-D and the occurrence of cancer, and positive results from epigenetic assays (e.g., Wigle et al., 1990; Figgs et al., 2000; Holland et al., 2002; McDuffie et al., 2001). The International Agency for Research on Cancer (IARC) has classified 2,4-D as a possible human carcinogen while the U.S. EPA has classified it as a Group D not classifiable as a human carcinogen. The EPA last evaluated 2,4-D in 1989 but it is scheduled for re-evaluation in 2003/2004 (U.S. EPA, 2002).

b) Glyphosate: Glyphosate, the active ingredient in the product Roundup®, is a broad-spectrum, non-selective herbicide that is used on golf courses for spot treatment of weeds. Glyphosate is absorbed through foliage and soft tissue of the plant and acts by inhibiting amino acid metabolism of the shikimic pathway throughout the plant (Tomlin, 1997). Glyphosate strongly adsorbs to moist soil and has a reported half-life ranging between 1 and 174 days (PIP Glyphosate, 1996).

There is evidence for and against the genotoxic potential of glyphosate. Clements et al. (1997) reported that tadpoles exposed for 24 hours to approximately 1.5 mg/l of Roundup experienced no DNA damage whereas concentrations of 6.75 and 27 mg/l resulted in significant DNA damage over controls. Peluso et al. (1998) reported that mice intraperitoneally injected with 400, 500 or 600 mg/kg of Roundup (commercial formulation containing glyphosate and the surfactant polyoxyethyleneamine (POEA)) formed DNA adducts in the liver and kidneys in a dose-dependent manner, but no adducts were observed when only glyphosate was given. These results appear to indicate that POEA, and not the active pesticide ingredient, may have genotoxic potential. Rank et al. (1993) found that Roundup and glyphosate were non-genotoxic using the mouse bone marrow micronucleus test, and by using a weight-of-evidence approach resulting from an extensive review of the literature, Williams et al. (2000) concluded that glyphosate was non-genotoxic. Glyphosate is classified as an U.S. EPA Group D not classifiable as a human carcinogen. The U.S. EPA has initiated a reevaluation of the health effects of exposure to glyphosate scheduled for completion by 2003 (U.S. EPA, 2002).

3. Insecticides

a) Diazinon: Diazinon, the active ingredient in products such as Diazinon®, Basudin®, and Knox Out®, is a nonsystemic organophosphorous insecticide. Diazinon has a half-life in soil of between 2-4 weeks (PIP Diazinon, 1996). In water, diazinon's half-life is related to pH, with faster breakdown related to increasing pH. In neutral conditions, half-life can be as long as 6 months (PIP Diazinon, 1996). Although use of diazinon on golf courses and sod farms in the United States was banned in 1988, primarily due to its high toxicity and lethality to birds (PIP Diazinon, 1996), diazinon is still widely used in Canada though several bird kills have been reported (Frank et al. 1991). However, in conjunction with the Pesticide Management Regulatory Agency of Canada (PMRA), the manufactures of diazinon in Canada (Novartis and Makhteshim) have voluntarily agreed to discontinue commercial class diazinon products for lawn and turf use, with a full phase out by 2003/2004 (PMRA REV2000-08).

Diazinon has been shown to be non-mutagenic in both in vivo and in vitro mutagenicity assays (U.S. EPA, 2000). Research by Bianchi-Santamaria et al. (1997) indicated that diazinon produced a statistically significant increase in micronucleated human lymphocytes in vivo, but not in a dose-dependent manner. In CD-1 mice no significant change in micronucleated erythrocytes was observed in male and females given either 60 or 120 mg/kg diazinon by gavage (U.S. EPA, 2000). Kuroda et al. (1992) reported that diazinon did not induce sister chromatid exchange in vitro in Chinese hamster V79 cells at 0.05 to 0.4 ug/ml, nor did it change the mitotic and second mitotic index more than controls. Based on the available negative evidence, the U.S. EPA classifies diazinon as a Group E not likely human carcinogen (U.S. EPA, 2000).

SUMMARY

Many municipal governments throughout Canada are implementing, or are in the process of drafting bylaws, that regulate the use of pesticides with the purpose of protecting the health of citizens and the environment. A main health concern of the politicians and their constituents is the occurrence of cancer in relation to pesticide exposure. Opponents of these bylaws argue that there is a lack of scientific evidence linking pesticide exposure to poor health. Determining a clear, causal relationship between pesticide exposure and health problems such as cancer is extremely complicated and even the most unbiased and stringent studies cannot account for all variables. Although not always true, it appears that the association or lack there of, between pesticide exposure and human and animal cancer as reported in the epidemiological literature, is often related to the type of study being conducted. Case-control studies generally seem to indicate relationships between pesticide exposure and cancer far more than cohort studies (Dich et al. 1997). Case-control studies are often criticized for reporting increased risks because of potentially biased recall of exposed individuals or their respective respondents. Individuals may want to lay blame on a specific cause for their condition (e.g., pesticide exposure), and as such tend to over-report their exposures compared to controls. Cohort studies are generally criticized for underestimating risk because of poorly selected cohorts and short follow up times, especially when the time between exposure and the manifestation of the disease under study can be very long. The study by Lynge (1998) is an obvious exception, with cancer incidence in the cohort being measured for almost fifty years. Lack of information on the nature, amount, frequency, and timing of exposure can hamper the detection of cancer risk

in both types of studies. Even in laboratory studies, where basic genotoxic and epigenetic effects can be tested without confounding variables, studies do not show unequivocal results (see Table 1). Outcomes of studies can be influenced by the concentration of pesticide given, route of administration (i.e. inhalation, gavage, etc.), animal model (e.g., dog, rat, mouse) or cell type.

There appears to be convincing in vitro and in vivo laboratory and epidemiological evidence to support the claim that under certain circumstances, iprodione, chlorothalonil, PMA and 2,4-D have been associated with cancer in humans and animals. Carcinogenicity from these pesticides seems to be related to genotoxic and epigenetic effects. As is pointed out by some opponents of pesticide bylaws, although some studies may find associations between cancer and exposure to pesticides, these associations are usually very weak. In some cases this is true. Hayes et al. (1991) found that dogs whose owners used 2,4-D or hired lawn care companies to treat grass had dogs with canine malignant lymphoma 1.3 times more often than dogs whose owners used no pesticides. In fact, this small association between exposure and effect is one of the critiques of the Hayes et al. (1991) study (Carlo et al. 1992). Though the magnitude of this relationship is not as great as that reported by Hardell et al. (1981), where people exposed to phenoxyacetic acids and chlorophenols had a 6-fold greater risk of developing NHL and Hodgkin's disease than the reference population, an association existed nonetheless. Weak associations are very much different than no association at all, and studies presenting these results should not simply be disregarded or misinterpreted as meaning that exposure to the compound in question is not related to any health concern (Hayes 1987). Weak associations should be used to highlight concern over

the effects of exposure to the chemical or chemicals under study and lay the foundation for future work.

At the present time, I am unaware of any ongoing studies on health outcomes of animals or people that are likely to come into contact with these common golf course pesticides. Indeed, golf course professionals appear under represented in occupational studies dealing with the effects of long-term pesticide exposure. Pesticide application on golf courses is related to factors such as ambient temperature, humidity, sunlight, soil type, and superintendent preference (Beard 1989). Considering that iprodione, chlorothalonil, PMA and 2,4-D are not restricted to the Ottawa/Gatineau region, there will be areas of the world where golf courses have been built where growing conditions will necessitate the use of these pesticides for up to 12-months of the year. As a result, golf course superintendents, their staff, and the wildlife found on golf courses could be exposed to these individual pesticides, and mixtures of these pesticides, for extended periods of time. Aside from the study by Kross et al. (1996) where a link between cancer-related PMR and pesticide exposure was presented (but was not directly measured due to study design constraints), researchers interested in pesticides and golf courses have not written about health effects related to exposure. Rather studies have focused on pesticide runoff, the accumulation and movement of pesticide residues in soil, and contamination of ground water by pesticides (Matthews et al. 1995; Ryals et al. 1998). In view of the fact that there is *in vitro* and *in vivo* laboratory and epidemiological evidence suggesting that under certain circumstances these commonly used golf course pesticides have been associated with the onset of animal and human cancer, I suggest that there is a need to conduct research into the health effects of those individuals likely to be chronically exposed to these pesticides. Golf course

superintendents, their staff, and the wildlife found on golf courses are likely candidates for such studies.

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Table 1. : Summary of case-control and cohort studies dealing with the relationship between cancer and pesticide exposure. Abbreviations: Non-Hodgkin's lymphoma (NHL), Hodgkin's disease (HD), small lymphocytic lymphoma (SLL), soft-tissue sarcoma (STS), odds ratio (OR), risk ratio (RR), proportionate mortality ratio (PMR). P values are given to indicate a significant difference between cases or cohorts and a control group.

Study	Type	Cancer	Statistic	Exposure	Pesticide
Hardell et al. 1981	Case-control (general population)	NHL, HD	6 fold greater risk than control	Various possible exposure periods	Phenoxyacetic acids
Zahm et al. 1990	Case-control (farmers)	NHL	2 fold greater risk than controls	<21 days/yr	Phenoxy herbicides
Zahm et al. 1990	Case-control (farmers)	NHL	7 fold greater risk than controls	>21 days/yr	Phenoxy herbicides
Waddell et al. 2001	Case-control (farmers)	SLL	OR= 2.8	Life-time exposure	Diazinon
Waddell et al. 2001	Case-control (farmers)	NHL	p>0.05	Life-time exposure	Diazinon

McDuffie et al. 2001	Case-control (farmers)	NHL	OR=1.96	Life-time exposure	Dicamba
McDuffie et al. 2001	Case-control (farmers)	NHL	OR=2.2	Life-time exposure	Mecoprop
McDuffie et al. 2001	Case-control (farmers)	NHL	OR=3.4	Life-time exposure	Aldrin
Lynge 1999	Cohort (herbicide factory workers)	Numerous	p>0.05	Up to 4 yrs	Phenoxy herbicides
Lynge 1999	Cohort (herbicide factory workers)	STS	p<0.05	Up to 4 yrs	Phenoxy herbicides
Zahm 1997	Cohort (pesticide applicators)	Mortality	p>0.05	Various possible exposure periods	Mixtures
Wigle et al. 1990	Cohort	Mortality	RR= 1.3	Applied	Mixtures (mainly 2,4-D)

	(farmers)			pesticides to 100 acres	
Wigle et al. 1990	Cohort (farmers)	Mortality	RR=1.9	Applied pesticides to 100-249 acres	Mixtures (mainly 2,4-D)
Wigle et al. 1990	Cohort (farmers)	Mortality	RR=2.2	Applied pesticides to >250 acres	Mixtures (mainly 2,4-D)
Kross et al. 1996	Cohort (golf course superintendents)	Mortality (brain cancer)	PMR= 234	Various possible exposure periods	Mixtures
Kross et al. 1996	Cohort (golf course superintendents)	Mortality (NHL)	PMR=237	Various possible exposure periods	Mixtures

Table 2.: Studies indicating positive (+) or negative (-) in vitro or in vivo mammalian genotoxic potential of the commonest active ingredients used in golf courses. Abbreviations: DNA strand breaks (SB), unscheduled DNA repair (US), micronucleus occurrence (MN), sister chromatid exchange (SCE), chromosomal aberrations (CA), gene mutation (GM) (e.g. forward, reverse, specific mutation), DNA adducts (A), in vitro study with metabolic (S9) activation (†), in vitro study without metabolic (S9) activation (‡), in vivo study (*), active ingredient (a.i.), per os (p.o.), intraperitoneal injection (i.p.), Chinese hamster ovary cell (CHO), lowest effective dose (LED), highest ineffective dose (HID), phenylmercury acetate (PMA), 2,4-dichlorophenoxyacetic acid (2,4-D).

Compound	Study	Detail	Form of Pesticide	Species (cells)	Endpoint	Result (LED, HID)
Chlorothalonil	Lodovici et al. 1997	* (in feed)	a.i.	Wistar rats (liver cells)	A	+ (0.09 mg/kg/d)
	Lebailly et al. 1997	‡	a.i.	Humans (lymphocytes)	SB	+ (1hr: 50 μM, 24 hr: 10 μM)
	Godard et al. 1999	*	a.i.	Sprague-Dawley	SB	-

		(p.o.)		rats (tissues)		(2000 mg/kg)
	Godard et al. 1999	‡	a.i.	Hamster (CHO)	SB	+ (0.5 µmol/l)
	Galloway et al. 1987	†, ‡	a.i.	Hamster (CHO)	CA	+ (0.5 µg/ml)
	Galloway et al. 1987	†	a.i.	Hamster (CHO)	SCE	+ (2.5 µg/ml)
	Galloway et al. 1987	‡	a.i.	Hamster (CHO)	SCE	- (2.5 µg/ml)
	McGregor et al. 1988	‡	a.i.	Mouse (tk+/tk- lymphoma cells)	GM	+ (0.12 µg/ml)
PMA	Lee et al. 1997	‡	a.i.	Human (lymphocytes)	SCE	+ (10 µM)
2,4-D	Adhikari & Grover 1988	* (i.p.)	a.i.	Wistar rats (bone marrow)	CA	+ (35 mg/kg)

	Ahmed et al. 1977	†, ‡	a.i.	Human (SV-40 fibroblast)	US	- (1000 μM)
	Charles et al. 1999a	‡	a.i. and salts	Han Wistar rats (hepatocytes)	US	- (478 μg/ml)
	Charles et al. 1999a	*	a.i. and salts	Han Wistar rats (hepatocytes)	US	- (1000 mg/kg)
	Charles et al. 1999b	* (gavage)	a.i. and salts	CD-1 mice (bone marrow)	MN	- (542 mg/kg)
	Gollapudi et al. 1999	†, ‡	a.i. and salts	Sprague-Dawley rats (lymphocytes)	CA	- (5000 μg/g)
	Gollapudi et al. 1999	†, ‡	a.i. and salts	CHO	GM	- (5000 μg/g)
	Jenssen & Renberg 1976	* (i.p.)	a.i.	CBA mice (bone marrow)	MN	- (1 hr/7 d after 100

						mg/kg injection)
	Korte & Jalal 1982	‡	a.i.	Human (lymphocytes)	SCE	+ (10 µg/ml)
	Linnainmaa 1984	*	Vesakontu ho Tasku®	Human (lymphocytes)	SCE	- (10.99 mg/l)
	Pilinskaya 1974	* (oral)	a.i.	Mice (bone marrow)	CA	+ (100 mg/kg)
	Pilinskaya 1974	‡	a.i.	Human (lymphocytes)	CA	+ (0.02 µg/ml)
Glyphosate	Clements et al. 1997	* (immersed)	Roundup®	Tadpoles	SB	+ (6.75 mg/l)
	Li & Long 1988	†, ‡	a.i.	CHO	GM	- (20 mg/ml)
	Li & Long 1988	‡	a.i.	Fischer 344 rat (hepatocytes)	US	- (0.125 mg/ml)
	Li & Long 1988	*	a.i.	Sprauge-Dawley	CA	-

		(i.p.)		rats (bone marrow)		(1000 mg/kg)
	Peluso et al. 1998	* (i.p.)	Roundup®	CD 1 mice (kidney, liver)	A	+ (600 mg/kg)
	Peluso et al. 1998	* (i.p.)	a.i.	CD 1 mice (kidney, liver)	A	- (270 mg/kg)
	Rank et al. 1993	* (i.p.)	Roundup®	NMRI-Bom Mice (bone marrow)	MA	- (200 mg/kg)
	Rank et al. 1993	* (i.p.)	a.i.	NMRI-Bom Mice (bone marrow)	MA	- (200 mg/kg)
Diazinon	Bianchi-Santamaria et al. 1997	†	a.i.	Human (lymphocytes)	MN	+ (0.04 µg/ml, not dose dependent)
	Chen et al. 1981	‡	a.i.	CHO	SCE	-

						(80 μg/ml)
	Chen et al. 1982	†	a.i.	CHO	SCE	- (80 μg/ml)
	Kuroda et al. 1992	‡	a.i.	CHO	SCE	- (0.4 μg/ml)
	Lopez & Carrascal 1987	‡	a.i.	Human (lymphocytes)	CA	+ (30 μg/ml, for 36 hr)

CHAPTER 3:

USE OF NON-DESTRUCTIVE BIOMARKERS TO MEASURE EFFECTS OF PESTICIDE EXPOSURE IN MEADOW VOLES (MICROTUS PENNSYLVANICUS) LIVING IN GOLF COURSE ECOSYSTEMS OF THE OTTAWA/GATINEAU REGION

INTRODUCTION

The causal relationship between acute pesticide exposure and mortality in non-target wildlife is well documented. In 1995-1996, over 20,000 Swainson's hawks (*Buteo swainsoni*) died in Argentina after they had fed on grasshoppers recently exposed to the insecticide monocrotophos (Woodbridge et al., 1995; Hooper et al., 1999). Similarly in the United States and Canada, American Widgeons (*Anas americana*) and Canada Geese (*Branta canadensis*) were found dead on golf courses after feeding on turf that had been treated with the insecticide diazinon (Frank et al., 1991; Kendall et al., 1992). In fact, mortality of wildlife species frequenting agricultural landscapes is thought to be a regular and frequent occurrence with the current suite of insecticides registered in North America (Mineau et al., 1999; Mineau 2002).

Unlike these clear causal relationships, the association between sub-lethal pesticide exposure and chronic health problems (e.g., cancer, birth defects, endocrine disruption) are not as easily established. In most cases chronic health problems have long latency periods and may take many years to manifest themselves. Thus, historical and current exposure of wildlife to anthropogenic chemicals is needed to determine a relationship between exposure and chronic effects. Unlike human case-control studies where historical exposure is determined through detailed questionnaires of "cases" or their proxies, it is virtually impossible to obtain historical exposure records for wildlife. What is more, many wildlife species are highly mobile and may be exposed to pesticides or other chemicals at many different sites, making exposure assessment at their capture site that much more difficult.

Golf courses and the meadow voles that live within them are excellent models to measure the long term, chronic effects of sub-lethal exposure to pesticides. As mentioned previously, present day golf course superintendents have many pesticide products available to them to help maintain a well-groomed and healthy turf. A survey of golf courses in Alberta by the Alberta Environmental Protection Office indicated that fungicides made up the majority of pesticides applied to golf courses (64%), followed by herbicides (34%) and insecticides (2%) (AEP 1998). As reported in a document published by the Attorney Generals Office of New York State, golf courses on Long Island used almost four to seven times the average amount of pesticides, on a kg/ha basis, than was used for agricultural purposes (Koppell 1994). Thus, the unintentional exposure to pesticides of non-target animals living in golf courses is extremely likely. Moreover, superintendents generally keep detailed records of their pesticide applications (e.g., where and when products are applied, what was applied, etc.) so exposure assessment can be conducted on wildlife presumably in contact with these chemicals.

The majority of studies that have investigated the use of pesticides on golf courses have not focused on health issues. Rather, studies have focused on pesticide runoff, accumulation and movement of pesticide residues in soil and the contamination of ground water by pesticides (Kenna 1995; Matthews et al., 1995; Ryals et al., 1998). I am unaware of any ongoing studies on the chronic health outcomes of animals or people that are likely to come into contact with pesticides on golf courses, even though there appears to be increasing potential for their unintentional exposure. Accordingly, the purpose of this field study was to conduct a biomonitoring study to measure the effects of pesticide exposure in meadow voles living in golf course ecosystems. Specifically, measurements of

genotoxicity (e.g., DNA strand breaks and micronuclei formation), developmental instability (e.g., fluctuating asymmetry (FA), congenital birth defects (e.g., skeletal terata)), clinical haematology (e.g., differential blood counts), general body condition (e.g., body mass-length relationships), and blood parasite load (e.g., *Trypanosoma sp.* and *Bartonella spp.*) were assessed in voles captured on golf courses of the Ottawa/Gatineau region of Canada.

MATERIALS AND METHODS

Animal Sampling and Handling

Attempts at capturing meadow voles took place from 15 June to 21 July 2001, from 01 September to 10 October 2001 and from 21 May to 30 September 2002 at six golf courses (designated A through F) and two reference sites from the Ottawa/Gatineau region of Canada. From 08 June to 18 June 2003 animals were trapped only at the reference sites. Voles were captured in live traps (Sherman PLFAHD perforated traps (9cm x 3cm x 3.5cm), Tallahassee, FL, USA) baited with either a 20 to 25g peanut butter-molasses-oatmeal bar or organic apple slices. Traps (n=50-80 depending on golf course) were set just before sunset in a grid pattern at a minimum of two different meadows within the golf course, adjacent to greens, tees or fairways, and checked the following morning approximately one hour after sunrise (refer to Appendix II for exact capture dates and capture sites). During nights where the ambient temperature was below 10° C or rain was expected, traps also contained a square of Nestlet bedding material (Ancare, catalogue

#NES3600). Traps were not set when night time temperatures were below 4° C due to the high risk of trap mortality (Courtin et al., 1991).

Captured voles were returned to a work site on the golf course and emptied from their trap into a Ziploc® bag (27 cm x 28 cm) and anaesthetized using isofluorane (isofluorane set at 4-5 % of total volume (volume/volume) with oxygen flow rate of 1.0 L/min). Once sedated, animals were placed dorsally on a table and anaesthetic was continuously administered via a nose cone (isofluorane set at 2-3 % of total volume (volume/volume) with oxygen flow rate of 1.0 L/min). Between 50 and 100 μl of blood was collected from the jugular or carotid arteries, or via a non-lethal cardiac puncture, using a 25-gauge needle attached to a 1cc pre-heparinized syringe. White blood cells were used for the comet assay and differential blood smears, immature red blood cells (polychromatic erythrocytes, PCE) were used to detect micronuclei, and whole blood was used in parasitology analysis. In 2003, blood was not obtained from reference animals. After collection, blood was emptied into a microcentrifuge tube and placed in a cooler of crushed ice. Standard length (i.e., tip of nose to base of tail) was measured to the nearest 0.1 cm using digital callipers (Mitutoyo Absolute Digimatic Callipers) and mass obtained to the nearest gram with a 30 g Pesola balance. Dorsal-ventral and lateral radiographs were taken using a portable x-ray machine (#HF8015, Minxray Inc., (Northbrook IL, USA); 50mA, 15KVp, 0.04 sec exposure, 24 inch focal plate-film distance) with Kodak MG-1 green sensitive, high-speed film. These setting were selected after I conducted trials using many different setting combinations and determined that these setting produced the strongest contrast and clarity in radiographs. Measurements of FA and skeletal birth defects were measured from radiographs (Figure 2). All captured individuals were marked

for later identification by numerical ear punching so they would not be mistakenly analyzed again.

Following these procedures, animals were placed in clean traps containing food and allowed to recover from the anaesthetic. Once fully awake, individuals were released at their exact site of capture. All traps that contained animals were returned to the laboratory and washed using antibacterial dish soap and warm water and allowed to air dry. The Animal Care Committee of the University of Ottawa approved handling and treatment procedures, and scientific permits for capturing animals were issued by the Ontario Ministry of Natural Resources (Authorization No.: 2001-14 (2001), 1000441 (2002), 1007568 (2003)) and Quebec Société de la Faune et des Parcs (Authorization No.: 01051001207SF (2001), 02030105007SF (2002)).

Pesticide and Metal Analysis

Superintendents from each course provided a copy of their pesticide use records for each research season (2001, 2002). Based on this information, total pesticide use (kg/m^2), days since the last application of a pesticide to the study area and the amount of the last pesticide applied to the study area (kg/m^2) were determined for each product on an active ingredient (a.i.) basis. Historically, organochlorine pesticides (OC) and certain metals (e.g., mercury, lead and cadmium) were used on golf courses. Because of their persistent properties, these compounds may still be present and capable of biological action. To provide signature levels of residues and putative exposure levels for each course, body burdens of these compounds were measured in three animals from each course, euthanized

while under anaesthetic. Individuals were euthanized in the fall during times of high population densities to ensure that destructive sampling did not result in population damage, a likely occurrence if sampling were to take place in the spring, when surviving over-wintered populations could be low. Animals were stored at –40°C in hexane-rinsed glass jars with Teflon-lined lids and OC pesticides and metal residues were measured in pooled samples of individuals from each of the golf courses. OC pesticides were measured in liver, total mercury in kidney, muscle and fur, cadmium in kidney, and lead from bone, at the National Wildlife Research Centre, Canadian Wildlife Service (Ottawa, ON, Canada). Samples for OC pesticides analysis were cleaned through neutral extraction, Gel Permeation chromatography, and by Florisil column chromatography. Quantitative analysis was performed using capillary gas chromatography. Samples for metal analysis were freeze-dried and total mercury was determined (without acid digestion) on an AMA-254 mercury analyzer, and cadmium and lead levels were determined using graphite furnace atomic absorption spectrophotmetry with Zeeman background correction. Quality assurance samples were analyzed along with the samples. Detailed analysis techniques can be obtained in methods manuals MET-CHEM-OC-04C, MET-CHEM-AA-03E, and MET-CHEM-AA-02E, from the National Wildlife Research Centre, Canadian Wildlife Service (Ottawa, ON, Canada).

Alkaline Comet Assay

The alkaline comet assay was conducted according to the method of Singh et al. (1994), as modified by McNamee et al. (2000). In the laboratory and under subdued

lighting, 20 μl of whole blood from each individual was diluted with 180 μl of calcium- and magnesium-free phosphate buffered saline (**PBS**-diluted 1:10). A 30μl aliquot of the diluted cell suspension was added to 270 μl of liquefied 0.75% low melting point agarose (~44° C) and mixed gently by pipetting. A 120 μl aliquot of the blood-agarose mixture was cast into an individual well of a 2-well Lab-Tek II chamber (Nalge Nunc, catalogue # 154461-B), affixed to a 6 cm x 10 cm piece of GelBond acetate film (Mandel, catalogue #53740). Human blood obtained through finger prick (L Knopper) was simultaneously run and used as an internal control, once per course in 2001 (collected over a two week period), and on each GelBond film containing vole blood in 2002. Once the blood-agarose suspension solidified, the entire GelBond film was placed in 50 ml lysing solution (2.5M NaCl, 10 mM tetra-sodium ethylene diamine tetra acetic acid (EDTA), 10mM Tris base, 1% n-lauryl sarcosine, 1% Triton X-100; pH 10) overnight at 4° C. In 2001, lysis buffer contained 10% dimethyl sulfoxide (**DMSO**), but this was not added in 2002 because it was found that DMSO can alter the formation of comets due to its membrane stabilizing effects. GelBond film was removed from the lysis buffer, gently rinsed with doubly distilled H_20 for approximately 30 seconds, and placed in 100 ml of alkaline electrophoresis buffer (0.3M NaOH, 10 mM tetra-sodium EDTA, 0.1% (w/v) 8-hydroxyquinoline, 2% (v/v) DMSO; pH 13.1) for 30 minutes. Following this, the GelBond film was placed in a gel electrophoresis apparatus (Horizon 58 Life Technologies BRL) containing 100 ml of fresh electrophoresis buffer and electrophoresed at 27 volts (constant voltage) for 20 minutes (e.g., 1.5V/cm, ~350-400mA). The GelBond film was then gently rinsed in doubly distilled H_20, placed for 30 minutes in 50 ml of neutralizing solution (1 M ammonium acetate; pH 7.0), rinsed again in doubly distilled H_20, then allowed to dehydrate for approximately two

hours in 100% ethanol. Films were hung to air dry, and subsequently stored in manila envelopes.

In 2001, blood from four voles was exposed to varying levels of ^{137}Cs g-radiation (0, 0.1, 0.3, 0.5, 1.0, 2.0 Gy) (GammaCell 40, Atomic Energy of Canada Ltd, Canada; 0.987 Gy/min) to serve as a positive control and in order to generate a dose-response curve in the comet assay (see below). Immediately after radiation exposure, the vole blood samples were mixed with agarose, cast onto Gelbond film and processed for the alkaline comet assay as described above.

At a later date, the GelBond films were cut into three strips, each containing two individual gels, and stained for 10-15 minutes in SYBR Gold (1/10,000 dilution of stock SYBR Gold with doubly distilled H_2O: Molecular Probes catalog #S-11494). GelBond strips were double rinsed in water and placed onto a glass microscope slide, covered with a cover slip, and gently pressed with a cloth to remove excess water. Comet images were captured with a Hitachi CCD Color Camera (KP-D581) and a Matrox Comet card (Matrox Electronic Systems, Montreal, PQ, Canada) and analyzed using Alkomet version 3.1 image analysis software at 330X magnification. Tail length is defined as the distance from the leading edge of the comet head to the leading edge of the tail. Tail moment is defined as the tail ratio (i.e., the relative fluorescence intensity in the comet tail region, to that in the entire comet) multiplied by the distance (μm) between the centers of gravity of the head and tail regions (Hellman et al., 1995). Comets that had tail lengths or moments greater than 4 SD from the average were not included in the calculation of the mean tail and moment for each individual (McNammee et al. 2000).

Cell Viability

The viability of lymphocytes in whole blood was determined via the dual stain viability assay (Strauss 1991) by mixing 50 μl of diluted whole blood (1:10 with PBS) with 50 μl of cell viability solution (1.2 ml PBS, 50 μl ethidium bromide (0.2 mg/ml), 1.2 μl stock fluoroscene diacetate (5 mg/ml acetone)). Diluted blood was added to the viability stain in a small tube wrapped in aluminum foil. Approximately 12 μl of the mix was loaded onto both sides of a hemacytometer and viable cells (e.g., fluoresced green) and non-viable cells (e.g., fluoresced red) were manually counted. Samples with viability below 95% were discarded from further analysis.

Micronucleus Assay

In 2002, blood smears for the micronucleus assay were made on glass microscope slides with approximately 3 μl of undiluted whole blood. Smears were made with a sub-sample of the same blood used for the comet assay. After air-drying, smears were fixed for five minutes in 100 ml of 100% methanol and then allowed to air dry (Hayashi et al. 1983; Krishna and Hayashi 2000). Micronucleated polychromatic erythrocytes (MN-PCE) and normochromatic erythrocytes (NCE, mature red blood cells) were scored after blood smears were stained with acridine orange (0.1 mg/ml H_20) for 60 seconds and double rinsed. The percentage of MN-PCE in 2000 PCE was counted using a 60X magnification oil immersion lens, and the percentage of PCE out of 1000 NCE was determined from digital images taken of the smear. Approximately 100 to 150 NCE were observed on each digital image

and cells were generally counted on the fringe of the blood smear were there was no cell overlap.

Fluctuating asymmetry

Fluctuating asymmetry (FA) is defined as random deviations from perfect bilateral symmetry with no genetic predisposition towards one side or the other being larger (Palmer and Strobeck 1986). FA was determined by measuring the lengths of the right and left humerus, ulna, ilium, femur and tibia from dorsal-ventral radiographs taken of voles in all three years. All measurements were taken using digital callipers (Mitutoyo Absolute Digimatic Calipers) that were connected to a desktop computer, and the data automatically entered onto a spreadsheet program. Callipers were zeroed before new measurements were taken on a different animal. I conducted all measurements blindly, and repeated measurements of the traits were taken on the same day, but without the knowledge of the previous value.

If the difference between repeated measurements for each trait was ± 3 standard deviations from the mean of that trait, then the measurement was considered an outlier. I believe that these values are not indicative of true measurement error, especially since they were so far removed from the average difference between repeated measurements, and any vole that had an outlier associated with it was removed from subsequent analyses. To calculate the measurement error associated with each trait, replicate right and left side measurements for each vole were entered in a two-way mixed model analysis of variance, with "side" as the fixed variable and "individual" as the random variable (Palmer and

Strobeck 1986; Yezerinac 1992; Swaddle 1994; Møller and Swaddle 1997). Percent measurement error (%ME) was calculated using the following equation:

$$\%ME = [s^2_{within} / (s^2_{within} + s^2_{among})] \times 100$$

where $s^2_{among} = MS_{among} - MS_{within} / m$, where MS_{among} and MS_{within} are the mean square deviations among and within individuals respectively, and m is the number of repeated measurements (Yezerianc 1992).

FA associated with each trait was calculated as the mean right minus mean left measurement. Tests for skewness and kurtosis were conducted to determine the two statistical properties of FA: right minus left measurements must be approximately normally distributed and centred around a mean of zero (Møller and Swaddle 1997). Normal distributions are characterised by non-significant values of skewness (e.g., skewness / standard error of skewness (SSE) < 1.96, > -1.96) and kurtosis (e.g., kurtosis / standard error of kurtosis (KSE) < 1.96, > -1.96) (Sokal and Rohlf 1981).

The occurrence of FA is more reliably detected when analyses are based on the combination of multiple traits rather than individual traits alone (Palmer and Strobeck 1986; Leung et al. 2000). In some cases, not all of the five bones could be properly measured. Accordingly, I calculated two composite indices of FA: FA^CALL and FA^CIFT. FA^CALL is defined as the sum of absolute FA for all five traits divided by the number of traits measured, whereas FA^CIFT is the index based on the sum of absolute ilium, femur, and tibia, all divided by three.

Skeletal Terata

From the radiographs used for FA measurements, skeletal abnormalities indicative of congenital birth defects were quantified. Each radiograph was scanned using an Epson Perfection 3200 Pro colour scanner with the transparency unit adapter. Each radiograph was scanned with a density step filter and the scan exposure altered so pure white was set at 0% and pure black set at 100%. Thus, the more dense the bone, the lower the value. Using Adobe Photoshop 6.0, the opacity (i.e., density) of the right and left ilium and femur were determined by obtaining an average opacity of four 5 x 5 pixel sites within the area of the bone. To correct for base fog differences among the images, opacity values of bones were divided by the opacity value of an adjacent fogged area (i.e., air). The number of tail vertebrae and ribs was quantified and the occurrence of fused or forked ribs, or other major malformations identified (Manson 1982; Rogers and Kavlock 1996).

White Blood Cell Differential Count

Leukocyte differential counts are a standard diagnostic test to quantify changes in leukocyte levels. The differential count is the cornerstone in laboratory hematology and yields clinically useful information about organismal health and disease (Duncan 1994; Houwen 2001). Differential blood smears were made for voles captured in 2002, on double frosted bevelled microscope slides (Surgipath, Winnipeg, MN, Canada), with 1.5 μl of whole blood and allowed to air dry. Smears were couriered to Vitatech Laboratories (Markham, ON, Canada) on the day of collection for analysis by a veterinary pathologist

(Dr. M. Tant). A manual white blood cell (WBC) count was generated from air-dried blood smears by 1) counting ten fields at forty times magnification along the length of the smear, 2) calculating an average, and 3) multiplying the average by a microscope calibration factor (Tvedten 1988). Because of an uneven distribution of leukocytes on the smears (a result of the smearing technique), three readings were made from different areas on each slide, and an average of the three readings (for a total of 30 fields) was used as the uncorrected WBC count. A standard hundred-cell differential was performed (Tvedten 1988), and the number of nucleated red blood cells was counted. The WBC count was then corrected for the presence of nucleated red blood cells, and the absolute values for the differential were calculated. When there was no featheredge present, the uncorrected WBC count was estimated as three times the WBC of the body of the smear, based on the pattern of data from those slides that had an adequately feathered edge. A recent publication (Pap 2002) has raised concerns about the repeatability of leukocyte counts for low-density white blood types using this technique. Pap (2002) found that neutrophils and lymphocyte densities for individuals were highly repeatable from smear to smear, but the repeatability of monocyte, eosinophil and basophil counts was poor. Thus, only total number of lymphocytes and neutrophils were used in statistical analyses for this study.

Body Condition Index

Indices of body condition were based on the residuals of a linear regression of body mass on body length (e.g., tip of nose to base of tail). With this index, individuals with positive residuals are considered to be in better condition than predicted for their size, with

the opposite true for individuals with negative residuals (Krebs and Singleton 1993; Schulte-Hostedde 2001; Blackwell 2002). The regression of $\log_{10}$ mass on $\log_{10}$ length (R^2=0.85, p<0.0001) yielded a better fit than the regression of mass on length (R^2=0.80, p<0.0001), so I have used the $\log_{10}$- $\log_{10}$ regression for the basis of the body condition index for voles captured in all three years (as suggested by Krebs and Singleton 1993). Pregnant females were excluded from the analysis.

Blood Parasites

In 2002, blood smears were made on glass microscope slides with approximately 3 μl of undiluted whole blood. After air-drying, smears were fixed for five minutes in 100 ml of 100% methanol and then allowed to air dry. At a later date blood smears were stained with acridine orange (0.1 mg/ml H_20) for 60 seconds and double rinsed in H_20. The occurrence and quantity of *Trypanosoma sp.* (Figure 3) but only the occurrence of *Bartonella spp.* (Figure 4), were quantified by visually scoring fifty fields of view in the feathered edge of the blood smear where these was no overlap of blood cells, using a sixty times magnification oil immersion lens (approx. 30,000 red blood cells).

Statistical Analyses

Comet assay: The relationship between comet assay parameters in relation to daily climate parameters (e.g., maximum, minimum and mean daily temperatures and humidity for the day prior to capture and on the day of capture—refer to appendix III for weather data) was

determined using linear regression analysis. ANOVA was performed to determine if there were relationships between tail length and moment and vole **reproductive status** and sex.

ANOVA was conduced to determine whether or not tail length and moment varied between 2001 and 2002, and to determine if they varied in relation to capture site, current use application parameters like the number of days since the last application of a pesticide, and capture site-days since last application interaction. Results from these analyses indicated that there were no golf course by days since last application effects (see below), and considering that voles from each golf course were captured at different sites throughout the course (refer to appendix II capture dates and areas), and because pesticide applications on golf courses were not homogeneous in respect to time and areas applied to (i.e., pesticides applied on the tee box of one hole were not necessarily applied on the green of another hole), I consider the voles captured for this study to be independent samples because each individual would have its own exposure regime.

The relationship between comet assay parameters and the total amount of pesticide application (a.i./m^2) in or around the area of capture up to the capture date in 2001 was determined using linear regression analysis. Due to the non-linear relationship between tail length and moment in relation to the number of days since the last application of a pesticide and since the last application of a known genotoxic pesticide in 2002 (see below), non-linear regression analysis ($Y=Ae^{-bx} + C$) were performed. Values of A and b (the y-intercept and slope, respectively) for the non-linear regression were obtained from the linear regression of log (Y-C) against days since the last application of a pesticide or since the last application of a genotoxic pesticide. C represents the lowest value of Y since it reaches an asymptotic nadir above zero. The relationship between tail length and moment

and the last amount of pesticide containing a potentially genotoxic active ingredient applied to the capture site (e.g., dose-response relationship) for both years was determined using a re-randomization test (Collins 1994).

Micronucleus assay: The relationship between micronucleus assay parameters in relation to daily climate parameters (e.g., maximum, minimum and mean daily temperatures and humidity for the day prior to capture and on the day of capture—refer to appendix III for weather data) was determined using linear regression analysis. Because micronuclei are quantified in PCE up to 120 days since their formation due to genotoxic damage in erythrocyte stem cells, I used a nested ANOVA to compare micronuclei levels among capture sites (golf courses and references sites) rather than in relation to current use application parameters.

Organismal Effects: ANOVA was performed to determine if there were relationships between the measured organismal biomarkers and vole reproductive status and sex. FA and skeletal birth defects manifest themselves during development, but because I measured juvenile and adult voles, it was not possible to determine their exposure to pesticides *in utero*. Thus, I compared $FA^{C}ALL$ and $FA^{C}ITF$ and skeletal birth defects among capture sites (golf courses and references sites) using a nested ANOVA. Parametric tests like the ANOVA are valuable for testing differences between or among groups in regards to absolute values of FA because ANOVAs are reasonably robust with respect to modest departures from normality and homogeneous variances, and skewed data (as is the case with absolute FA) have very little effect on either the significance or the power of the test,

regardless of whether sample sizes are equal or not (Gangstead and Thornhill 1998). In the case of FA analysis, the use of non-parametric tests reduces power and increases type II error (Gangstead and Thornhill 1998). In fact, when analyzing FA data parametric tests are not only appropriate but they are preferred (Gangstead and Thornhill 1998; Palmer 2003).

Body condition, haematology and blood parasites can change with time so it is possible that these endpoints could be related to current pesticide use parameters (e.g., total amount of pesticide application, days since the last application of a pesticide). The relationship between these endpoints and the total amount of pesticide application (g a.i./m^2) in or around the area of capture up to the capture date, and the days since the last application of a pesticide, was determined using linear regression analysis. Nested ANOVA was used to ascertain if there were differences in these endpoints in relation to capture site (golf courses and reference sites).

Logistic regressions were used to determine the relationship between these pesticide parameters and *Bartonella spp.* infection. The difference in *Bartonella spp.* infection rates between voles captured on golf courses and at reference sites was determined using chi-square.

All statistics were conducted using SYSTAT ver. 10 for Windows, except for the chi-square analysis that was conducted in Microsoft Excel 2000 and the randomization test, which was conducted using a software program developed at the Canadian Wildlife Service (Collins 1994).

RESULTS

A total of 142 voles were captured during the course of this study. 29 voles were captured between two different reference sites and the remaining 113 voles were captured among the golf courses. In 2001, 24 females (19 non-reproductive, 5 reproductive) and 25 males (21 non-reproductive, 4 reproductive) were captured. In 2002, 32 females (22 non-reproductive, 10 reproductive) and 42 males (13 non-reproductive, 29 reproductive) were captured. In 2003, 7 females (3 non-reproductive, 4 reproductive) and 12 males (3 non-reproductive, 9 reproductive) were captured. Captures in relation to the number of days since a pesticide application in or around the area of capture ranged from 1 to 58 days, and in most cases (except for golf course E where animals were only captured at 58 days since the last application), there was variation at each site (Figure 5). In some cases it was not possible to measure all of the endpoints on an individual and as a result sample sizes vary among specific endpoints.

Pesticide and metal residues

Levels of OC pesticides were very low and invariable between golf courses. 1, 2, 3, 4- and 1, 2, 4, 5-tetrachlorobenzene, pentachlorobenzene, α, β, and γ hexachlorocyclohexane, hexachlorobenzene, octachlorostyrene, heptachlor expoxide, oxy-, trans-, and cis-chlordane, trans- and cis-nonachlor, dieldrin, photomirex, mirex, TCPM (tris (4-chlorophenyl) methanol), p, p'-DDT (dichlorodiphenyltrichloroethane) and p, p'-DDD (1, 1-dichloro-2, 2-bis (p-chlorophenyl) ethane) were either not detected (<0.0001 μg/g wet weight in livers) or only found in trace amounts (0.0001-0.0009 μg/g in livers). Residues of p, p'-DDE (1, 1-dichloro-2, 2-bis (p-dichlorodiphenyl) ethylene) were the highest of all

measured OC pesticides, but still relatively low, and had an average value of 0.04 μg/g. Levels of total mercury, cadmium and lead were also very low. Values of total mercury in kidney, fur and muscle ranged from less than 0.06 to 0.157 μg/g dry weight, cadmium levels in kidneys ranged from 0.46 to 1.57 μg/g dry weight and lead in the bone ranged from 0.08 to 3.3 μg/g dry weight.

Current use fungicides with the a.i. chlorothalonil and iprodione, followed by herbicides containing 2,4-dichlorophenoxyacetic acid (2,4-D) and glyphosate, and insecticides containing diazinon, were most commonly used on golf courses between 1999 and 2002. Of these a.i's., chlorothalonil appears to be the most likely to be capable of inducing *in vivo* genotoxicity (refer to Chapter 2). Less commonly used fungicides contained the a.i.'s quintozene, benomyl, and propiconazole, and less commonly used insecticides had the a.i.'s carbaryl and imidacloprid. On average, a total of 0.3 to 5.6 g a.i./m^2 was applied each year to the golf courses in this study (Table 3).

^{137}Cs Radiation Exposure

Both tail length and tail moment increased in a dose dependent fashion predicted by the mechanisms of the comet assay (Fairbairn et al., 1995; Collins et al. 1997) (Figure 6). Tail length increased rapidly at lower doses but then slowed and showed signs of reaching a plateau at higher doses. Tail length increased from an average of 17 μm at the control level to 52 μm at 1.0 Gy (a difference of 35um), but only increased to 72 μm with another doubling of dose (2.0Gy), a difference of 20μm. Conversely, tail moment increased slowly at low doses but appeared to increase at a greater rate at higher doses. Tail moment

increased from an average of 0.60 at the control level to 2.6 at 1.0 Gy (difference of 2.0), but increased to 6.5 with a doubling of dose (2.0Gy), a difference of 4.

The highest tail lengths and tail moments from wild caught voles were comparable to tail lengths and moments in blood exposed to approximately 1.0 Gy radiation. The observed damage in voles from golf courses is much higher than would be expected in control animals. Because of these suggestive results relating DNA damage to pesticide exposure, and the evidence of no handling effects (i.e., stable human values over time), the biomonitoring study was continued again in 2002.

Comet assay

Comet tail moments were statistically different ($p<0.0001$) between 2001 and 2002. In 2001, average tail moment was 0.7 whereas it was 1.6 in 2002. Average tail lengths were also lower in 2001 than in 2002 (17.9 and 21.5 μm, respectively), but they were not statistically different ($p=0.18$). Considering that DMSO acts not only as a radical scavenger but also as a membrane stabilizer, it can be expected that cells exposed to DMSO would have lower DNA migration than those not exposed to DMSO. The differences in tail moment and tail length between 2001 and 2002 are consistent with this explanation. Thus, data for each year are presented separately. Only one of the samples had a cell viability count below 95% and it was subsequently discarded from analysis.

2001

Human blood was collected over a two-week period and average tail length and tail moment from these samples was 13.1μm (10.5-16 μm) and 0.36 (0.31-0.42), respectively. The comet assay was conducted on twenty-two voles in 2001. Tail lengths and tail moments of vole blood were not related to the sex or reproductive status of captured voles, or to any daily climate parameter ($p>0.05$).

There was intra-golf course variation in tail length and tail moment, and no one golf course had animals that all had high levels of DNA damage (Figure 7a). The number of animals captured at each day since the last application of a pesticide varied among and between capture sites (Figure 5). Average tail length (means from each capture site) was not significantly related to the golf course from which animals were captured, nor was it related to the number of days since last application of a pesticide or to the golf course-days since last application interaction (Two-way ANOVA, p=0.14, 0.11, 0.57, respectively). There was very little variation in respect to captured animals in relation to days since last application. For example, voles from golf course A were captured approximately 8 and 10 days since last application of a pesticide at (Figure 7a), and voles from golf course D were only captured 58 days since the last application. However, voles were caught at four time points (5, 34, 35 and 36 days since last application) from golf course B, and inspection of the data indicates a decreasing trend in tail length with days (Figure 7a). Average tail moment (means from each capture site) was not significantly related to the golf course from which animals were captured or to the golf course-days since last application interaction, but was related to the number of days since last application of a pesticide (Two-way ANOVA, p=0.10, 0.05, 0.10, respectively). Considering that there was no golf course by

days since last application effects, I decided to continue analyzing the data using individual animals rather than golf course means.

Tail length (R^2=0.31, $p<0.01$) and tail moment (R^2=0.29, $p<0.01$), using individual voles from all capture sites together, significantly increased in a linear fashion in relation to the total amount of pesticide application (kg a.i./m^2) in or around the area of capture (Figure 8a). Tail length (R^2=0.36, $p<0.01$) but not tail moment (R^2=0.10, $p=0.15$) decreased in relation to the number of days since last application of a pesticide (Figure 9a). When regressed against the number of days since last application of Daconil®, the fungicide containing the genotoxic a.i. chlorothalonil (40.4%), both tail length (R^2=0.44, $p<0.001$) and tail moment (R^2=0.15, $p=0.05$) significantly decreased in a linear fashion (Figure 10a). Tail length and tail moment did not significantly increase in a dose-dependent manner ($p>0.74$ and 0.38, respectively) with the last amount of Daconil® applied (g a.i./m^2 basis) to the capture area (Figure 11a). However, an increasing trend was observed. Only one vole was captured at the low dose rate, and even though attempts were made, no animals were captured from reference sites. Visually it appears that the animals exposed to higher amounts (0.5 and 0.7 g a.i/m^2) had greater tail lengths and tail moments than the individual exposed to the lower amounts (0.08 g a.i./m^2).

2002

Unlike in 2001, human blood was collected the same day blood from voles was collected, and each was run simultaneously on the same GelBond film in the comet assay procedure. Unexplained variation occurred half way through the field season that caused an increase in tail length and tail moment in both control human and vole blood. This change

in damage in the control human subject was not related to any genotoxic producing activity (e.g., eating habits, smoking, intense exercise), and no biological reason was likely to cause such a dramatic difference in vole damage from all capture sites. Each step in the comet assay was reconstructed, post hoc, using new chemicals but the difference persisted. Since human control values were very stable before and after the increase, albeit around a different mean value, I felt confident using them as the basis for a correction factor to adjust vole data. Values of tail length and tail moment for post increased damage were adjusted by a ratio of that day's control blood value divided by the average value of control blood prior to the unexplained increase.

Sixty-nine voles were used for the comet assay in 2002. Sixty-one animals were captured at golf courses and the remaining 8 were from two different reference sites. Animals from reference sites were captured over a period of one month. Tail length and tail moment were not related to the sex or reproductive status of captured voles, or to any daily climate parameter ($p>0.05$).

There was considerable intra-golf course variation in tail length and tail moment, and no one golf course had animals that all had high levels of DNA damage (Figure 7b). The number of animals captured at each day since the last application of a pesticide varied among and between capture sites (Figure 5). Average tail length (means from each capture site) was significantly related to the golf course from which animals were captured, and to the number of days since last application of a pesticide but not to the golf course-days since last application interaction (Two-way ANOVA, $p=0.02$, 0.001, 0.64, respectively). Inspection of the data indicates that a greater number of voles captured at golf course A were found with high damage in the first few days since the last application compared to

other sites, and this is likely the reason for the observed golf course effect. When using data from this course alone, tail length is significantly related to days since last application ($p=0.004$). Average tail moment (means from each capture site) was not significantly related to the golf course from which animals were captured, to the golf course-days since last application interaction, or to the number of days since last application of a pesticide (Two-way ANOVA, $p=0.25$, 0.55, 0.75, respectively), but as with tail, when using data from golf course A along, moment is significantly related to days since last application ($p<0.001$, respectively). Considering that there was no golf course by days since last application effect, I decided to continue analyzing the data using individual animals rather than golf course means.

Neither tail length nor tail moment, using individual voles from all capture sites together, was significantly related to the total pesticide application (a.i./m^2) around the area of capture ($p=0.70$ and 0.35, respectively) (Figure 8b). Tail length did significantly decrease exponentially in relation to the number of days since last application of a pesticide ($R^2=0.29$, $p=0.001$) (Figure 9b) and in relation to the number of days since last application of Daconil® ($R^2=0.30$, $p<0.005$) (Figure 10b). Animals with the greatest tail lengths within the first six days of application had relatively low corresponding tail moments. This time-lag can be explained by the dynamics of the comet assay (see below). Animals with the greatest tail moments and with correspondingly high tail lengths were found 6 days after application (Figure 9b and 10b). Due to this time-lag effect, the relationship between tail moment and days since last application of pesticide was performed excluding values from days 1-5 in order to conduct the exponential regression analysis starting at the apex of damage. Tail moment significantly decreased exponentially in relation to the number of

days since last application of a pesticide (R^2=0.41, p<0.001) and the number of days since last application of Daconil® (R^2=0.42, p<0.001) (Figure 9b and 10b). Tail length and tail moment appeared to significantly increase (p<0.0001) in a dose-dependent manner in relation to the amount of Daconil® last applied (g a.i./m^2 basis) to the capture area (Figure 11b). Tail length measured at 0.5 g a.i./m^2 was significantly greater (p<0.05) than in voles from reference sites or those exposed to lower amounts.

Micronucleus assay

Percent MN-PCE was not significantly related to the sex or reproductive status of voles (p>0.50), or to any pesticide application parameter (e.g., total application, days since last application, etc.). Percent MN-PCE and total PCE were not significantly different among golf courses (p=0.47, p=0.14, respectively), nor were there significant differences between golf course voles and reference site voles (p=0.78, p=0.13, respectively). Voles captured at reference sites had an average of 0.2 % (0.0 - 0.4 %) MN-PCE and 4.8 % PCE (1.3 - 9.5 %) and voles captured at golf courses had an average of 0.1 % (0.0 – 0.6 %) MN-PCE and 4.4% PCE (0.4 - 13.9 %).

Fluctuating Asymmetry

Two FA^CALL data points were strong statistical outliers. Inspection of the radiographs of these animals suggests that they were positioned in a way that made certain bones on the right side appear larger than they actually were. Thus, these animals were removed from further analysis of FA. A total of 83 individual voles were measured for

asymmetry. All of the traits exhibited ideal FA. None had significant skewness or kurtosis (i.e., skewness / standard error of skewness (SSE) and kurtosis / standard error of kurtosis (KSE) were between 1.96 and -1.96), and all had a mean of approximately zero (Table 4). Percent measurement error was generally low and ranged from 0.6% to 9.0%, depending on which trait was measured. Percent measurement error of the ilium was lowest (0.6%) and that of the humerus highest (9.0%).

$FA^{C}ALL$ and $FA^{C}IFT$ were not related to the year in which animals were captured or to their sex or reproductive status (three-way ANOVA, $FA^{C}ALL$: p=0.28-0.63, $FA^{C}IFT$: p=0.39-0.56). Thus, animals from each site were pooled and $FA^{C}ALL$ and $FA^{C}IFT$ compared among sites. Neither $FA^{C}ALL$ nor $FA^{C}IFT$ were significantly different among sites (p=0.06 and p=0.55, respectively) (Figure 12).

Skeletal Birth Defects

A total of 79 individual voles were measured for skeletal birth defects. The number of caudal vertebra was not related to the sex or reproductive status of voles, or to the year of capture (three-way ANOVA: p=0.41, 0.53, 0.07, respectively). On average, voles from all three years had 17 caudal vertebrae; 3 animals had 15, 14 had 16, 45 had 17, 16 had 18 and 1 vole had 19 tailbones. The number of caudal vertebra was not related to the site in which animals were captured (p=0.18). All animals had 13 ribs and none were fused or forked. No other skeletal malformations were identified.

A total of 93 voles were measured for bone density. Right and left values were combined to calculate an average bone density for the ilium and femur. Bone density of

the ilium did not vary among year of capture or sex but was related to reproductive status (three-way ANOVA: p=0.08, p=0.71, p<0.005, respectively). Non-reproductive voles had an average density value of 0.77 (n=48, SE=0.009) and reproductive voles had an average of density value of 0.72 (n=45, SE=0.009), or about 5.0% denser. Ilium density in both reproductive and non-reproductive voles was not related to the area of capture (p=0.26, p=0.22, respectively) (Figure 13).

Bone density of the femur did not vary among year of capture or sex but was related to reproductive status (three-way ANOVA: p=0.80, p=0.77, p=0.006, respectively). Non-reproductive voles had an average density value of 0.84 (n=48, SE=0.008) and reproductive voles had an average of density value of 0.81 (n=45, SE=0.009), or approximately 3.0% denser. Femur density in both reproductive and non-reproductive voles was not related to the area of capture (p=0.21, p=0.20, respectively) (Figure 13).

Body Condition

Body condition was assessed on 125 voles. Body condition was related to year of capture, but not to reproductive status or sex (three-way ANOVA: p=0.05, p=0.10, p=0.20, respectively). The average body condition residual index in 2003 was 0.05 (n=18, SE=0.03), slightly greater than values from 2002 (index=0.01, n=49, SE=0.03) and 2001 (index=-0.02, n=58, SE=0.02). In general, voles (males and females combined) from golf courses had a mean condition value of -0.001 (n=109, SE=0.01) and voles from reference sites had a mean condition value of –0.006 (n=25, SE=0.03). Body condition was not related to the site of capture (p=0.89) (Figure 14), nor to the total amount of a.i. applied to

the capture site (R^2=0.006, p=0.53) or to the days since the last application of a pesticide (R^2=0.03, p=0.14). It is possible that other endpoints like FA, white blood cell levels and parasite load could be related to body condition. However, FA^CALL (R^2=0.008, p=0.51), FA^CITF (R^2=0.04, p=0.11), absolute white blood cell levels (R^2=0.03, p=0.18) and *Trypanosoma sp.* levels (R^2=0.05, p=0.61) were all unreliable predictors of body condition.

Haematology

Differential blood counts were conducted for 70 voles. Neutrophil levels (10^9 cells/L) were not related to sex or reproductive status (two way ANOVA, p=0.16, p=0.69). Lymphocyte levels (10^9 cells/L) were not related to sex or reproductive status, but were related to the sex x status interaction (two way ANOVA, p=0.27, p=0.65, p=0.003). Reproductive females males had an average of 3.6 x 10^9 lymphocytes/L (n=10, SE=0.81) and reproductive males had an average of 1.9 x 10^9 lymphocytes /L (n=27, SE=0.16). Lymphocytes levels in non-reproductive males and females were indistinguishable, with males having an average of 3.3 x 10^9 cells/L (n=12, SE=0.55) and females with an average of 2.5 x 10^9 cells/L (n=21, SE=0.23). Values of neutrophils and lymphocytes were not related to the total amount of a.i. applied to the capture site (R^2=0.02, p=0.24 and R^2=0.001, p=0.85) or to the days since the last application of a pesticide (R^2=0.07, p=0.14 and R^2=0.01, p=0.95), and did not vary between golf courses and reference sites (p=0.54 and 0.43, respectively) (Figure 15). Values of the complete differential count are presented in Table 5.

Blood Parasite Load

Only 9% of the 65 voles captured in 2002 were infected with Trypanosomes and parasitemia rates ranged from 1 to 74 Trypanosomes per 30,000 red blood cells. *Trypanosoma sp.* infection was not related site of capture (p=0.26), nor was it related to the total amount of a.i. applied to the capture area (R^2=0.004, p=0.63) or to the days since the last application of a pesticide (R^2=0.007, p=0.56). Eosinophil levels are considered the hallmark of parasitic and bacterial infection when elevated (Smith 1996). Eosinophil levels were not reliable predictors of *Trypanosoma sp.* infection (R^2=0.04, p=0.13).

50% of the 65 voles captured in 2002 had *Bartonella spp.* infection. *Bartonella spp.* infection was not significantly related to the total amount of a.i. applied to the capture area or to days since the last application of a pesticide (two-way logistic regression: p=0.76, p=0.17). The proportion of infected voles varied between golf course and reference animals, with 44% of voles infected from golf courses and 70% of voles infected from reference sites. However, the number of infected animals did not appear to be significantly related to capture site (x^2=2.4, degrees of freedom: 1, p=0.13). Eosinophil levels were also a poor predictor of *Bartonella spp.* infection (p=0.74).

DISCUSSION

Genotoxic Effects Of Pesticide Exposure

The observed DNA damage in these meadow voles was not related to body burdens of OC pesticides and metals historically used on these golf courses. Except for p, p'-DDE, OC pesticide residues were either not detectable or only found in trace amounts. Values of p, p'-DDE were the highest of all OC pesticides but were still in the range of previously published values for meadow voles from uncontaminated sites (reviewed in Talmage and Walton 1991). Reference values (dry weight) of cadmium in kidney range between 0 and 5.6 μg/g, lead in bone is approximately 12.0 μg/g, and mercury from numerous tissues is generally below 1.5μg/g (reviewed in Talmage and Walton 1991). In comparison, meadow voles from sewage-sludge treated fields had kidney cadmium levels between 1.4 and 23.0 μg/g, meadow voles from orchards had bone lead levels as high as 158.8 μg/g, and voles found in mercury contaminated sites had body burdens greater than 5.0 μg/g (reviewed in Talmage and Walton 1991). Similarly, metal residues were all within previously published reference ranges (reviewed in Talmage and Walton 1991). Considering that there was considerable intra-golf course variation in tail lengths and tail moments, and since no one golf course had animals that all had high levels of DNA damage, there appears to be nothing intrinsic about any of the golf courses that could cause genotoxicity (e.g., golf cart emissions, chemical spills, radon gas from geological features). Furthermore, since DNA strand damage was also not related to any climate parameters, the most likely cause of the observed genetic damage in voles is exposure to currently used pesticides on the golf courses.

Compared to pesticide use on golf course in the United States, application in the Ottawa/Gatineau region were quite low. On average, a total of 2.5 g a.i./m^2 was applied each year to the golf courses in this study. In a typical year in early 1990, golf courses in

the United States used approximately 6.0 g a.i./m^2 (Barbash and Resek 1996). In 2001, DNA damage was related to the total amount of a.i. applied, but was not in 2002. The most obvious difference between these years is sample size (22 animals in 2001 versus 69 in 2002) and variation in application rates, with a maximum of approximately 0.004 kg a.i./m^2 applied in 2001 but approximately 0.015 kg a.i./m^2 in 2002. Both of these differences are likely the reason for the observed positive relationship in 2001 (i.e., high type I error in 2001).

The most commonly used pesticides on the golf courses participating in this study were fungicides with the a.i.'s chlorothalonil and iprodione, herbicides containing 2,4-D and glyphosate, and insecticides containing diazinon. Of these five compounds, chlorothalonil is the one with the most evidence of in vitro and in vivo DNA damaging potential. Chlorothalonil is a broad spectrum, non-systemic foliar organochlorine fungicide. It has been shown to have genotoxic properties, generally only at low doses, and only during initial exposures, and chlorothalonil's genotoxic potential appears related to oxidative radical formation (Lebaily et al., 1997; Lodovici et al., 1997; Godard et al., 1999). These findings appear to coincide with the use pattern of chlorothalonil on these golf courses and with the observed DNA damage in meadow voles; that is relatively low levels of application are related to elevated DNA damage within the first six to ten days of application. It seems possible then that chlorothalonil is mechanistically capable of inducing the primary DNA damage observed in wild meadow voles.

Meadow voles from this study are most likely to come into contact with chlorothalonil containing pesticides by absorbing residues through the pads of their feet after walking on treated areas, from inhalation of drift after application, and orally after

ingesting treated items (e.g., turf grass, seed-pods, through grooming). The half-life of chlorothalonil on vegetation (is between 7 and 19 days (Elliott and Spurr 1993). This corresponds to an exponential decay slope of between -0.036 and -0.093. The slope of the exponential curve describing the relationship between tail length and tail moment and time since the last application of Daconil® in 2002 was -0.072 (95% Wald Confidence Intervals: -0.028, -0.117) and -0.087 (95% Wald Confidence Intervals: -0.048, -0.127), respectively. The slopes of these regressions were not significantly different from that of the decay slope of chlorothalonil on vegetation. Furthermore, tail lengths and tail moments significantly increased in a dose-dependent fashion in relation to the amount of Daconil® last applied to the capture area. In 2001 this was not the case likely due to low sample sizes and lack of variation in exposure rates, but the data were suggestive of a relationship. Even though the exact route of exposure was not measured in this study, these results suggest that oral exposure to chlorothalonil is a likely cause of primary DNA damage in these meadow voles.

Variation in tail length and tail moment at each time point after application appears to decrease with time (Figures 5-7). The most parsimonious explanation for this observation is differences in exposure to pesticides being applied. Just because voles were captured in an area of exposure doesn't necessarily mean that they were all equally exposed. It is possible that voles with high damage may have been on the grass at the time of application or had recently eaten pesticide treated grass, while those captured on the same day with low damage may have been deep in their burrows and not exposed. With time since last application, variation decreases because exposure rates decrease (pesticides disappear from the environment) and levels of DNA damage between "exposed" and

"unexposed" conspecifics become similar. It is also possible that voles with low damage have efficient DNA repair systems because of previous exposure to the pesticides applied (e.g., **hormesis**). This is especially likely if new voles (i.e., dispersing recruits from nearby fields or young voles) are encountering the pesticides for the first time. Voles naive to pesticides would be expected to show greater DNA damage than those previously exposed.

In 2002, animals with the greatest tail lengths within the first six days of application of a pesticide had relatively low corresponding tail moments. It was not until six days after the last application of a pesticide that voles with the greatest tail lengths also had correspondingly high tail moments. I did not feel confident concluding the same about 2001 because of the low sample size, even though the data appear suggestive of this time-lag relationship (Figure 6a and 7a). I am unaware of other field-based studies that have observed this time-lag effect between the apex of tail length and moment, but it can likely be explained by the dynamics of the comet assay mentioned previously. Indeed, the results from the ^{137}Cs exposure of vole blood lend support for this explanation. Tail length is expected to increase quickly with low levels of exposure to a genotoxin but plateau at higher exposures (Fairbairn et al., 1995). Tail moment is a product of tail length and the amount of DNA that has migrated into the tail. While DNA can only migrate so far due to electrophoretic conditions, the amount of DNA in the tail region can continue to increase as the dose increases. Thus, with increasing dose, it is tail intensity that increases but not tail length (Collins et al., 1997).

In relation to the data obtained from this present field study, tail length can be used to indicate initial DNA damage to while tail moment can be used to indicate the intensity of damage. Tail length in voles reached a maximum 1-day after application of a pesticide,

meaning that DNA loops, free ends or DNA fragments had migrated as much as possible. However, tail moments in these animals were still relatively low indicating that the amount of DNA damage was relatively low. Between days 1 and 5, damage was still occurring because even though pesticides were not reapplied, pesticide residues were still present. On day 6, animals reached the apex of tail moment meaning that the intensity of damage was highest at this time point, even though the original exposure to the pesticides was five days earlier. Thus, damage occurred within 24 hours of contact with a pesticide but was cumulative up to six days after initial exposure.

No chromosomal DNA damage was observed in these meadow voles as measured by the erythrocyte micronucleus assay. On average, meadow voles from golf courses had the same proportion of MN-PCE as voles from reference sites and these values fall within the range of normal basal levels of MN-PCE frequencies in laboratory mice and two other vole species. Abramsson-Zetterberg et al. (1997) reported that CBA laboratory mice, and wild-caught bank voles (*Clethrionomys glareolus*) and field voles (*Microtus agrestsi*) had basal MN-PCE frequencies between 0.09 and 0.21%. Red cell production, as indicated by the number of PCE, was not related to pesticide use either. On average meadow voles from golf courses had the same number of PCE as voles from reference sites. I have not found evidence to suggest that chlorothalonil has clastogenic potential. Even though chromosomal breaks are correlated with unrepaired and/or misrepaired DNA strand breaks (Fenech 2000), it appears that the meadow voles used in this study did not accumulate enough DNA damage to result in the formation of MN-PCE above basal levels.

Organismal Effects of Pesticide Exposure

Fluctuating asymmetry, skeletal birth defects, white blood cell levels, body condition, and parasite load have all been used as non-destructive biomarkers of organismal health in areas of pesticide use. For instance, greater FA than would be expected in animals from reference sites has been found in grey seals (*Halichoerus grypus*) and ringed seals (*Pusa hispida*) exposed to DDT and polychlorinated biphenyls (PCB) (Zakharov et al. 1997), in brown trout (*Salmo trutta*) living in polluted fluvial ecosystems (Sánchez-Galán et al. 1998) and to damselflies (*Xanthocnemis xealandica*) exposed to fungicides and insecticides (Hardersen 2000). Recent wildlife biomonitoring and human epidemiological studies have revealed a relationship between animals and humans living in areas of pesticide use and teratogenic outcomes. Ouellet et al. (1997) observed high rates of hindlimb deformities in frogs and toads living in agricultural sites exposed to pesticide runoff; Linzey et al. (2003) reported a correlation between body fat concentrations of p,p'-DDE and limb deformities in frogs and toads; and Bell et al. (2001) concluded that exposure to several classes of pesticides (e.g., organophosphates, pyrethroids, halogenated hydrocarbons, carbamates, and endocrine disruptors) during the 3rd to 8th week of pregnancy in humans was associated with fetal death due to congenital anomalies (e.g., anencephalus, hydrocephalus). Chronic exposure to pesticides has also been associated with health concerns like cancer and altered immune function. I also concluded from my literature review (Chapter 2) that there appears to be convincing *in vitro* and *in vivo* laboratory and epidemiological evidence to support the claim that under certain

circumstances, golf course pesticides like iprodione, chlorothalonil, phenylmercury acetate, and 2,4-dichlorophenoxyacetic acid, have been associated with cancer in humans and animals; Bishop et al. (1998) reported that tree swallows (*Tachycineta bicolor*) living in apple orchards experienced immunostimulation, whereas Linzey et al. (2003) reported depressed lymphocyte levels in amphibians in relation to pesticide exposure. In most cases parasite infection in rodent hosts is not considered to result in a pathogenic outcome, but in some cases *Trypanosoma* and *Bartonella* infection has been related to immunodepression (Ackerman and Seed 1976) and decreased reproductive function (Boulouis et al. 2001).

Unlike these studies, my results failed to suggest that voles from golf courses of the Ottawa/Gatineau region of Canada are less "healthy" than their conspecifics from reference sites. In general, the organismal biomarkers that were measured did not vary among capture sites or in relation to pesticide use parameters, and many of the endpoints were no different than what has been published for animals found living in reference sites. Though caudal vertebrae numbers did vary among voles, variation in vertebral numbers is not uncommon in small mammals. In CD-1 mice, under 5% of the population has less than 10 postsacral vertebrae, 30% have between 10 and 11, almost 65% have between 11 and 12, and less than 0.5% have more than 12 (Szabo 1989). Similar variation has been observed in thoracic and lumbar vertebrae in rabbits and laboratory rats (Manson et al. 1982; Rogers and Kavlock 1996). Levels of total white blood cells and levels of neutrophils, lymphocytes, monocytes, basophils and eosinophils for golf course voles and reference voles were generally indistinguishable, and were similar to unpublished differential blood count reports from a study of over 250 wild voles from Manitoba (S. Mihok, unpublished data). *Trypanosome sp.* and *Bartonella sp.* infection were well within previously published

values of infection levels. Woo et al. (1980) found that 2.7% of 374 meadow voles captured in southern Ontario were infected with *Trypanosome sp.* and Nelson (1949) found that 67% of 21 voles captured in Illinois were infected. Kosoy et al. (1997) captured seven species of rodents from the southeastern United States and found that 42.2% of 279 individuals were infected with some strain of *Bartonella.* Similarly, Birtles et al. (1994) found that 62.2% of 37 rodents captured in the United Kingdom were infected. My results also seem to suggest that infection with these organisms is non-pathogenic since eosinophil levels were not related to level of parasitemia or bacteremia. However, due to the potentially low repeatability of intra-individual eosinophil counts, the reliability of this relationship is limited.

Post-hoc power analyses were conducted to determine how many animals from each capture site would be needed to correctly accept the null hypothesis that no difference in measured biomarkers among capture sites existed, and to determine what the actual values of β (probability making a type II error, i.e., accepting the null hypothesis when it is false) were for each endpoint (Peterman 1990). To be 80% confident in my acceptance of the null hypothesis (with an α value of 0.05) that $FA^{C}ALL$ and $FA^{C}ITF$ were not significantly related to capture site (p=0.13, p=0.49, respectively), 113 and 469 voles would need to be captured per site. In relation to body condition and total white blood cell values, 64 voles and 103 voles, respectively, would need to be captured per site. Based on my actual sample sizes, values of β ranged from 60 to 75%. Thus, it is possible that true effects did exist (e.g., that $FA^{C}ALL$ is in reality related to capture site) but I was unable to detect the relationship due to low power likely caused by the small number of animals captured (Peterman 1990). Given my capture success and the relatively small size of capture areas,

it would not have been biologically possible to capture the statistical number of animals needed to minimize the type II error and maximize power associated with this biomonitoring study.

Several golf courses initially contacted for this study refused to allow me on site and it is therefore possible that my sample of courses was biased toward those with presumably lower pesticide use patterns. As mentioned above, DNA strand damage in voles was observed in relation to the number of days since the last application of a specific fungicide (Daconil®) and damage appeared to increase in a dose-dependent manner with the amount of the last application of Daconil®. It has been shown that chlorothalonil has genotoxic properties, but generally only at low doses, and only during initial exposures. These findings appear to coincide with the use pattern of Daconil® on these golf courses. It is possible that while low levels of pesticide exposure were able to initiate DNA strand breaks, they were not great enough to cause changes in developmental stability, body condition, blood levels or immune function. Unfortunately, I am unaware of studies that have measured these endpoints in meadow voles in areas of greater pesticide use, so no comparisons can be made to test this hypothesis.

Because asymmetry, birth defects, blood function, and parasitemia are related to survival, it is also possible that no relationship between these organismal endpoints and pesticide exposure was observed because animals with extreme abnormalities (e.g., high parasitemia, gross birth defects, large FA) may have been selected out of the population, and therefore, only healthy animals remained at the capture sites. In relation to developmental instability, it has been hypothesized that if FA is related to individual quality, then the most "unstable" individuals are the ones most likely to be removed from

the population during stressful conditions (Polak and Trivers, 1994). Though this hypothesis has been applied to FA, it is equally applicable for the other organismal endpoints measured in this study since they too are related to survival. There is considerable evidence for such "developmental selection" to occur. Allenbach et al. (1999) showed that mortality in fishes exposed to the pesticides lindane and parathion (given at the LC_{70} dose) was related to individual FA. Western mosquito fish (*Gambusia affinis*) and sand shiners (*Notropis ludibundus*) were used in a time-to-death in 96-hour toxicity test. Every three hours dead fish were removed from the aquaria and at the end of the 96 hours, the remaining fish were euthanized and FA measured in all animals. Results of the study indicated that the fish with the greatest average FA died earlier than fish with smaller average FA (Allenbach et al., 1999). Recently Polak et al. (2002) reported a reduction in FA in *Drosophila melanogaster* that was concomitant with high dose arsenite-driven mortality. If voles died *in utero* because of skeletal abnormalities, or died as juvenile or adults because of high parasite load or high FA, then the majority of voles remaining in meadows and being captured might have had the lowest FA, birth defect frequency, and parasite load in that population.

In general, results from this field study indicated that voles from golf courses did not appear to differ in their asymmetry, skeletal birth defects, blood parameters, parasite load, body condition or level of chromosomal damage when compared to voles from reference sites. However, voles from golf courses did appear to experience significantly greater DNA damage expressed as single or double strand breaks.

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Figure 2: Radiograph of two different meadow voles (Microtus pennsylvanicus) taken with a Minxray portable x-ray machine at 50mA, 15KVp, 0.04 sec exposure, 24 inch focal plate-film distance) on Kodak MG-1 green sensitive, high-speed film.

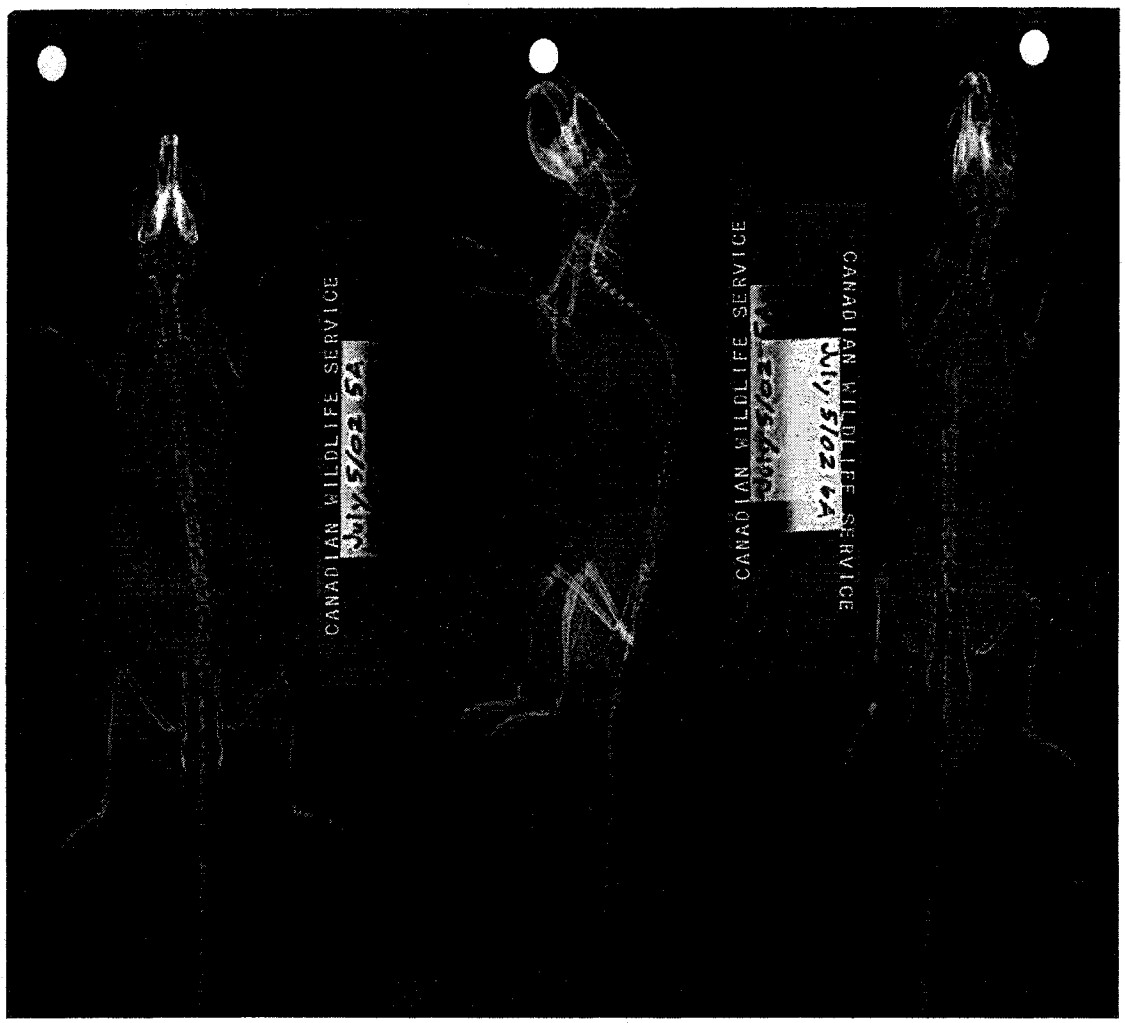


Figure 3.: *Trypanosoma microti* seen extra-cellularly in a whole blood smear of a meadow vole (*Microtus pennsylvanicus*). A white blood cell is observed in the upper right hand corner, and below it and to the left is a polychromatic erythrocyte (PCE). Blood smear has been stained with acridine orange.

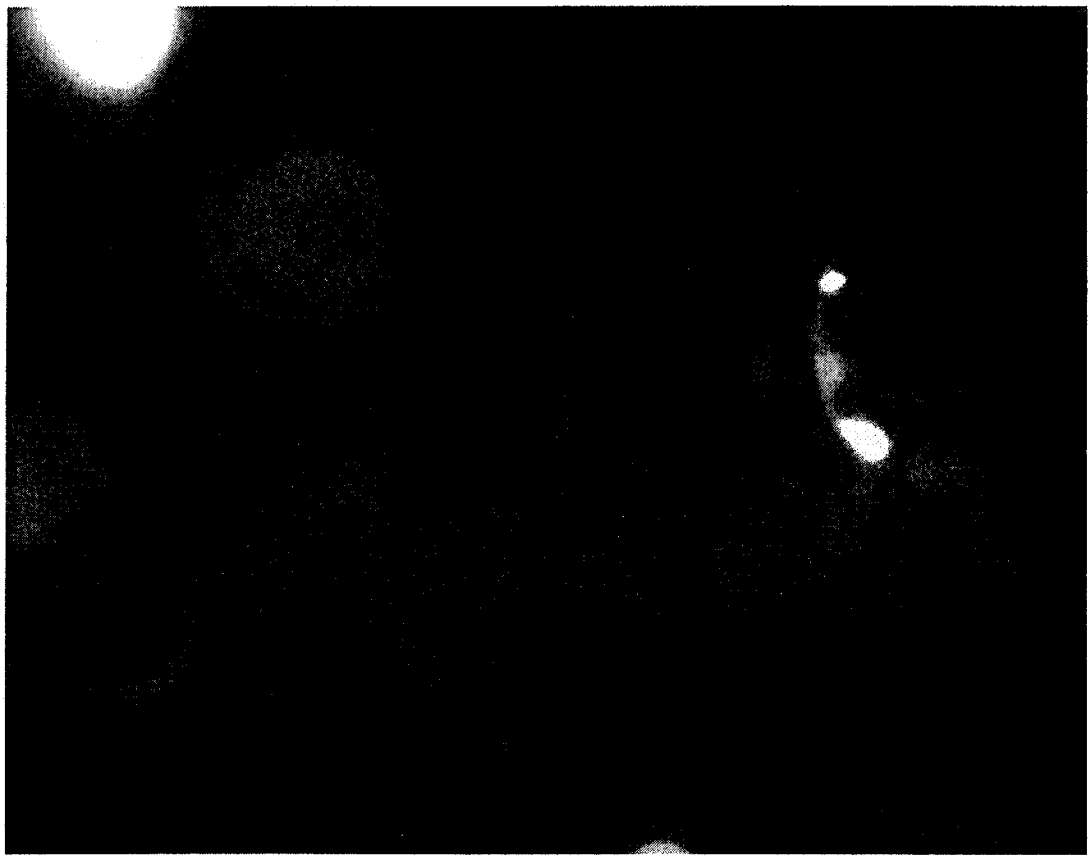


Figure 4.: *Bartonella sp.* seen intra-cellularly in whole blood smear of a meadow vole (*Microtus pennsylvanicus*). Blood smear has been stained with acridine orange.

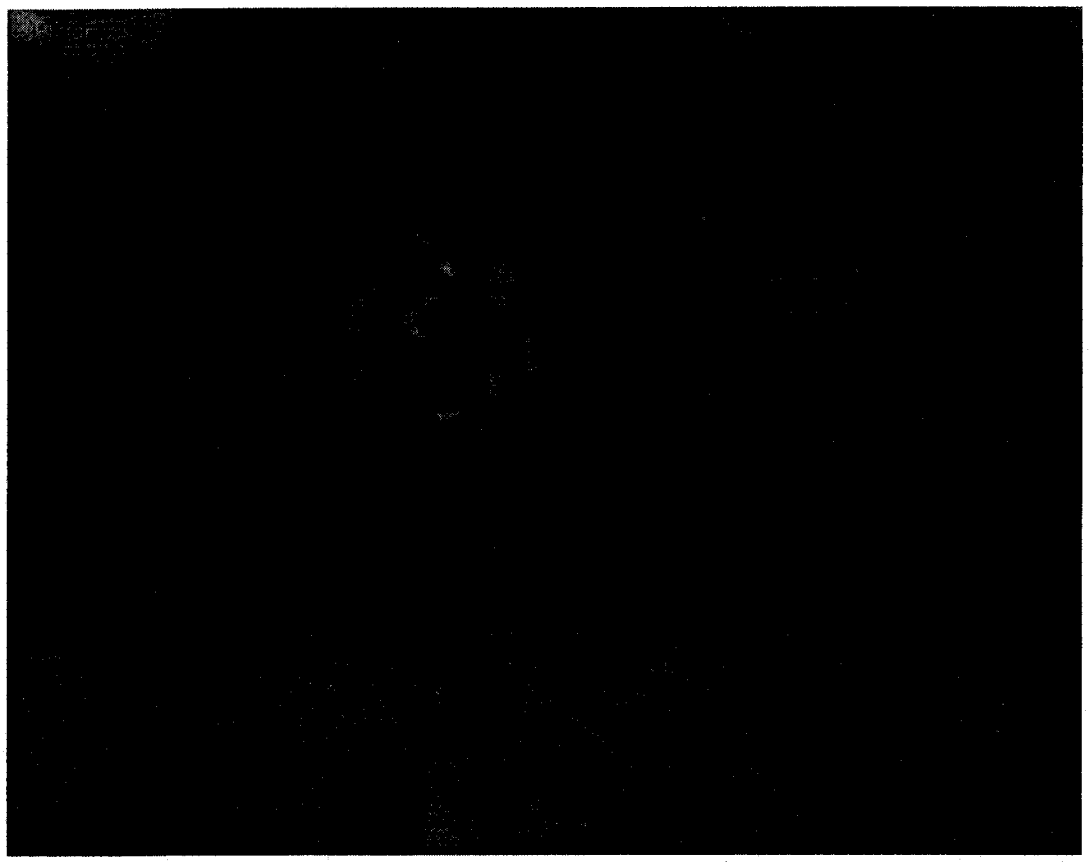


Figure 5.: Intra-site variation in the number of voles captured in relation to the number of days since last pesticide application for 2001 and 2002. Animals captured at day zero were captured at a reference site. Golf course A (●), Golf Course B (X), Golf Course C (+), Golf Course D (▲), Golf Course E (▼), Reference site (◀).

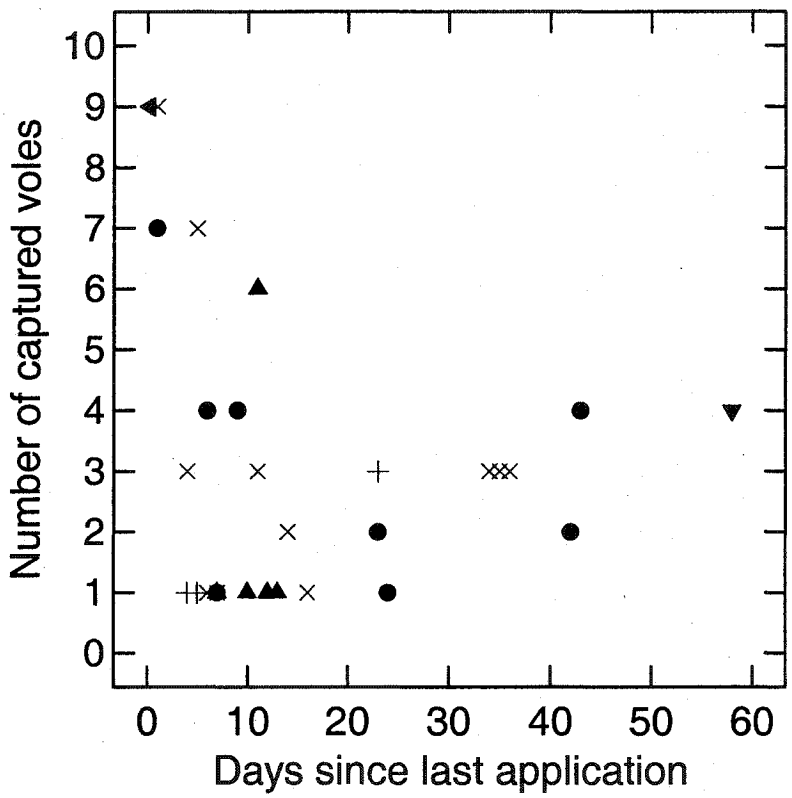
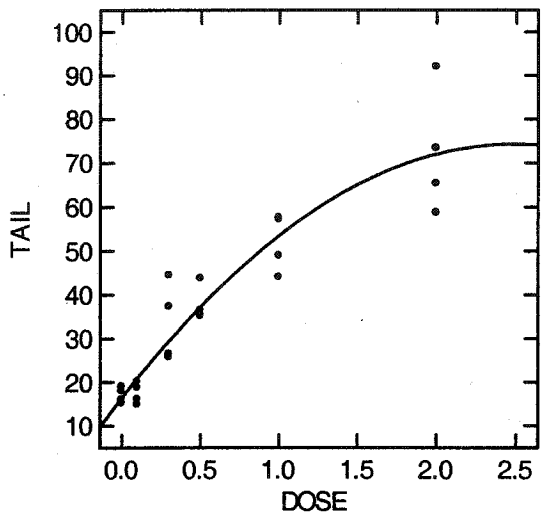


Figure 6.: Tail length (μm) and tail moment values from laboratory exposure of meadow vole (Microtus pennsylvanicus) blood to ^{137}Cs radiation.



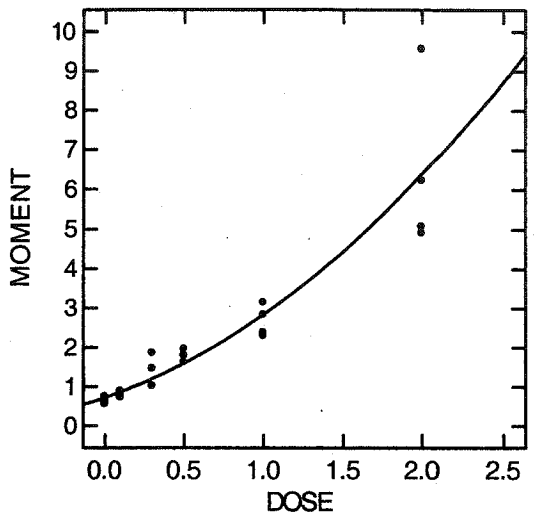


Figure 7.: Intra-site variation in tail length and tail moment in a) 2001 and b) 2002. Golf course A (●), Golf Course B (X), Golf Course C (+), Golf Course D (▲), Golf Course E (▼), Reference site (◀).

a.

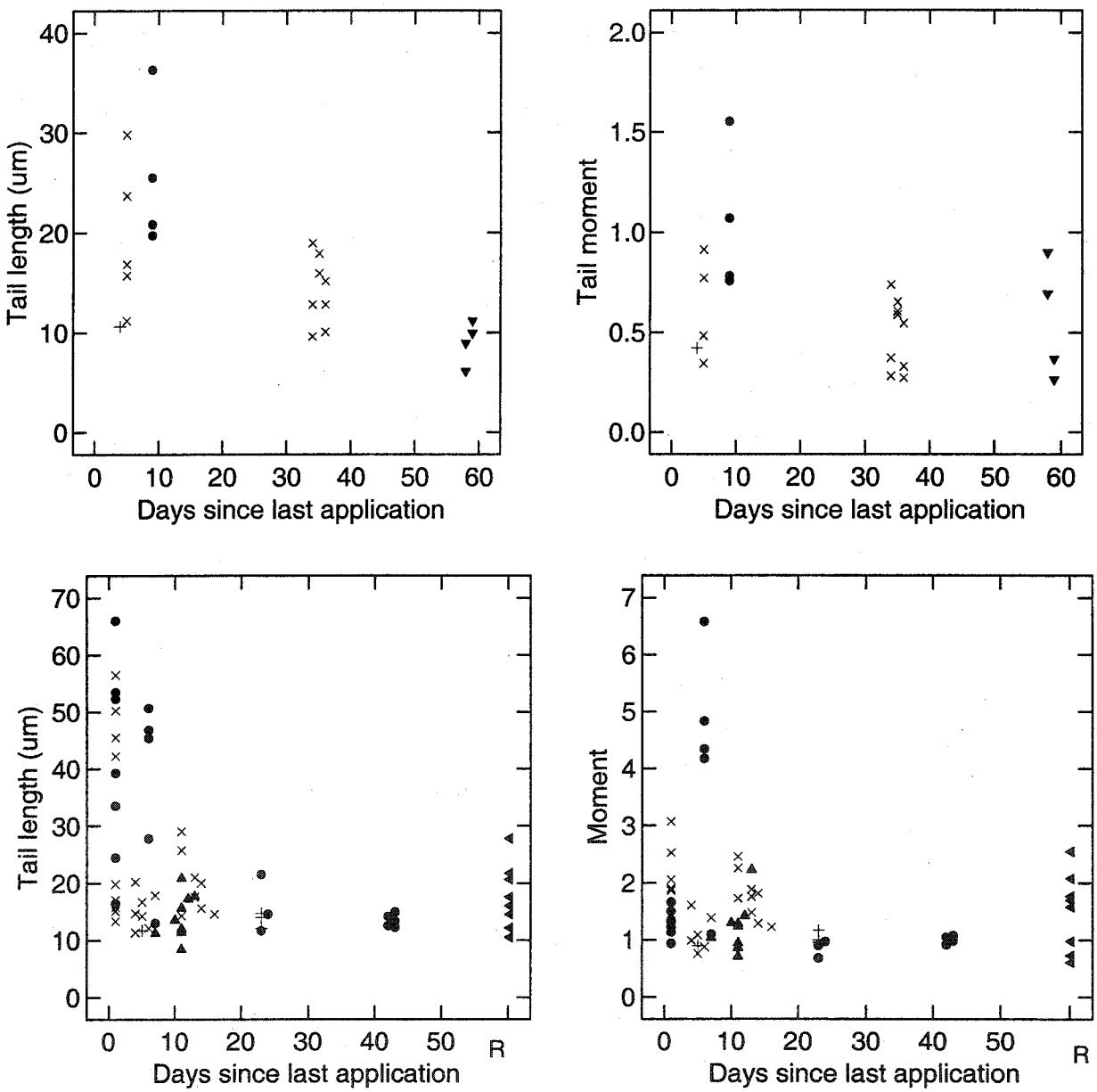
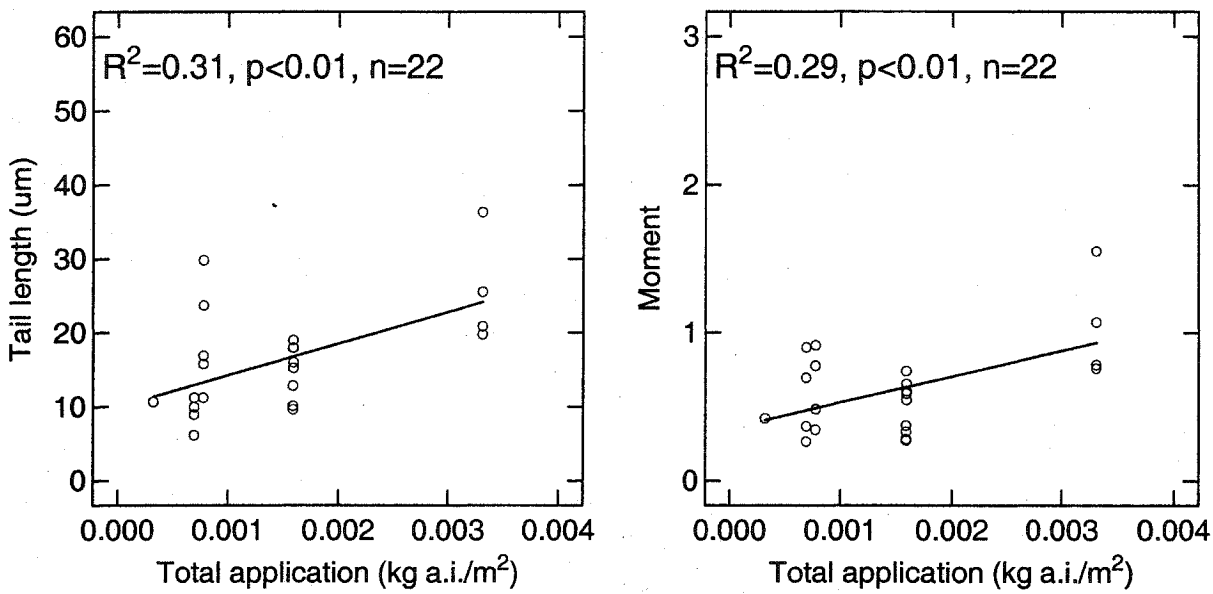


Figure 8.: Tail length (μm) and tail moment for meadow voles (*Microtus pennsylvanicus*) in relation to total application of all active ingredients (kg/m^2) up until the day of capture for a) 2001 and b) 2002.

a.



b.

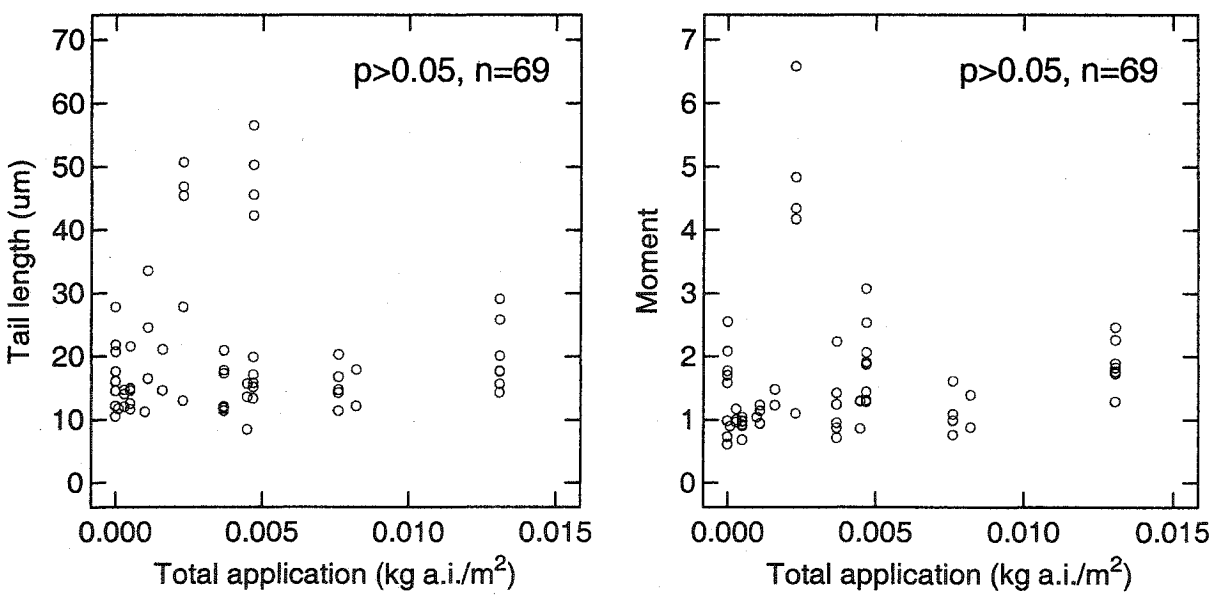
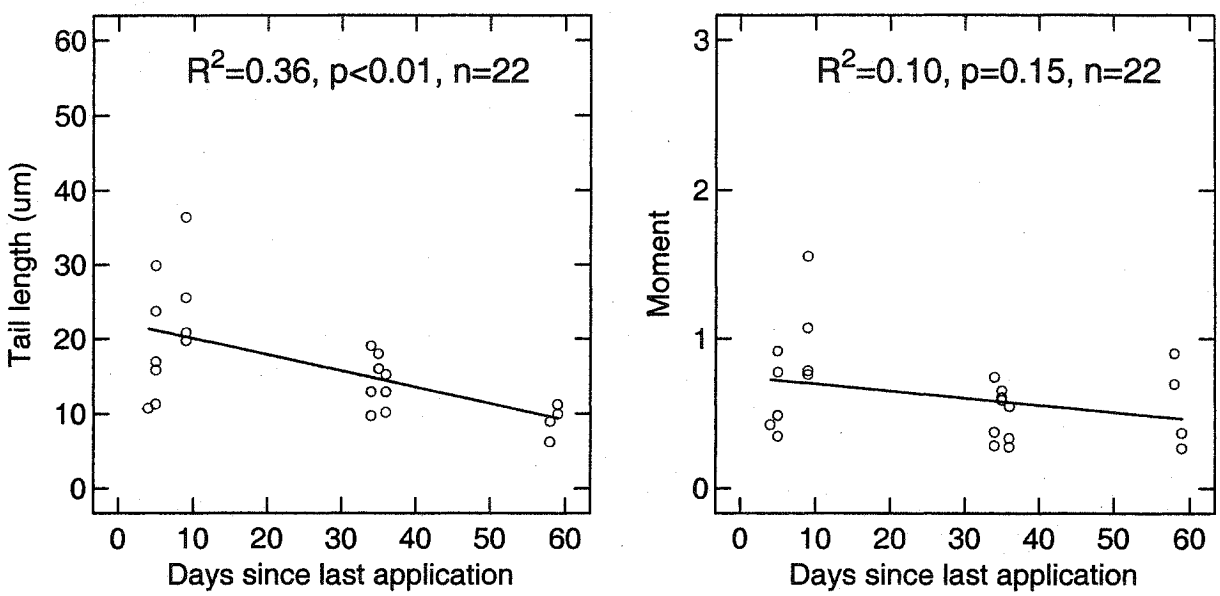


Figure 9. : Tail length (μm) and tail moment for meadow voles (*Microtus pennsylvanicus*) in relation to the number of days since last application of a pesticide up until the day of capture for a) 2001 and b) 2002. "R" on the x-axis indicates values from reference animals.

a.



b.

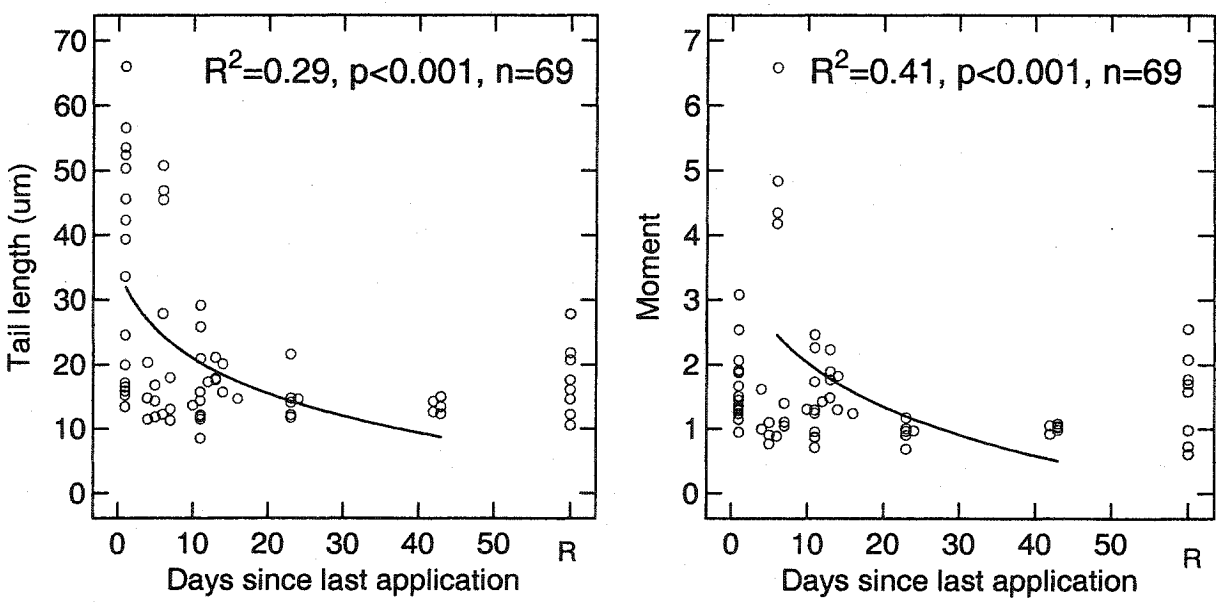
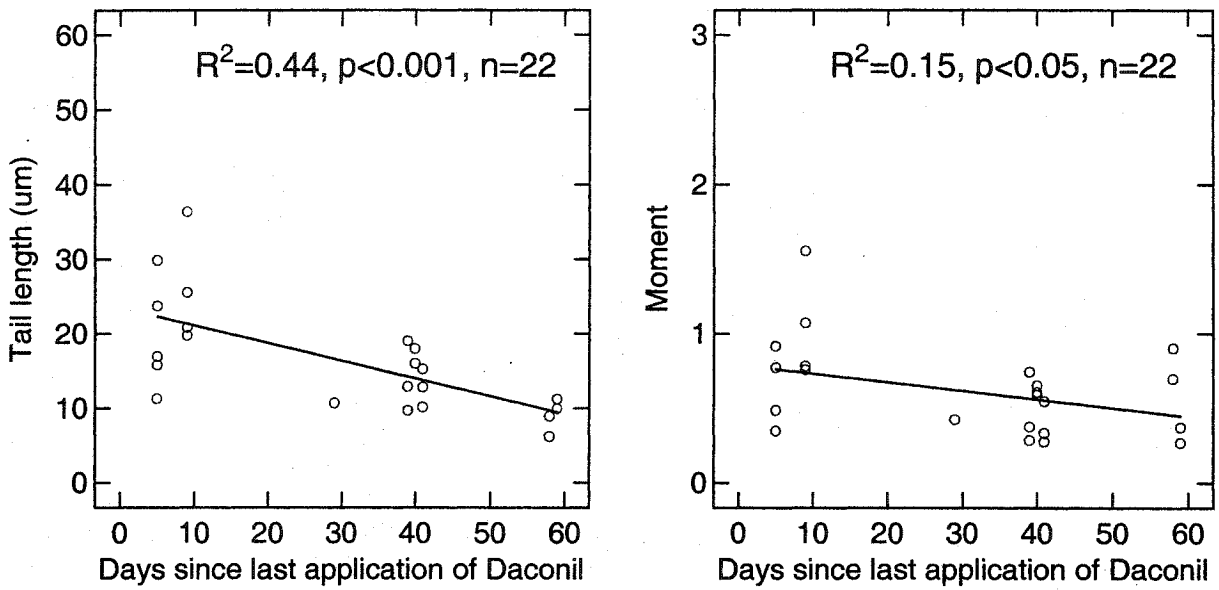


Figure 10. : Tail length (μm) and tail moment for meadow voles (*Microtus pennsylvanicus*) in relation to the number of days since last application of Daconil® up until the day of capture for a) 2001 and b) 2002. "R" on the x-axis indicates values from reference animals.

a.



b.

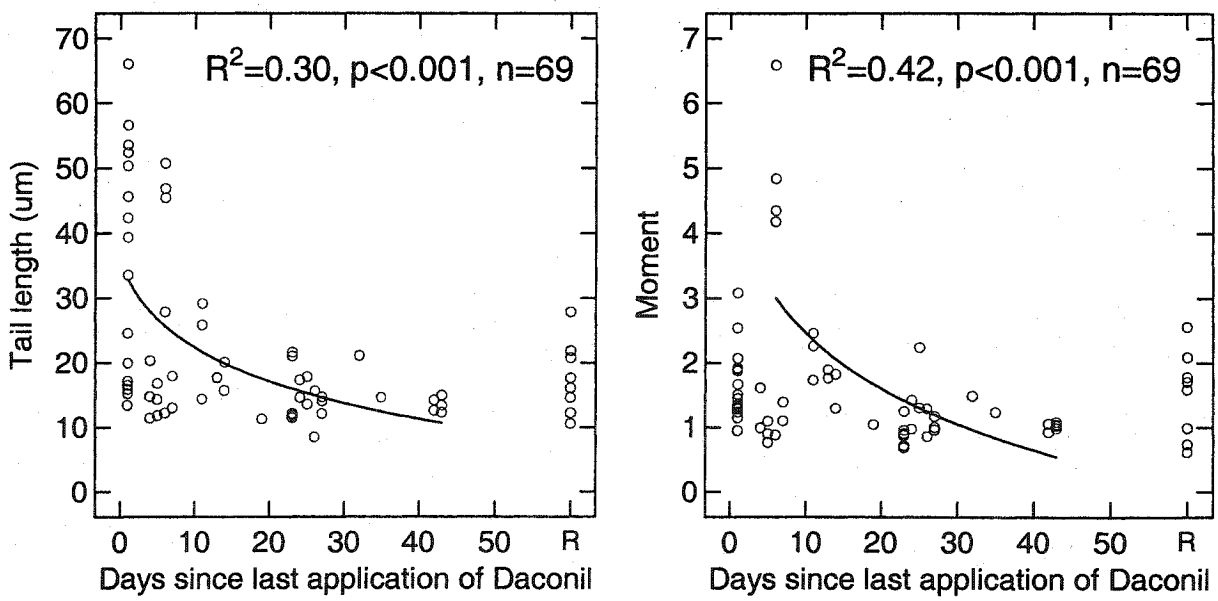
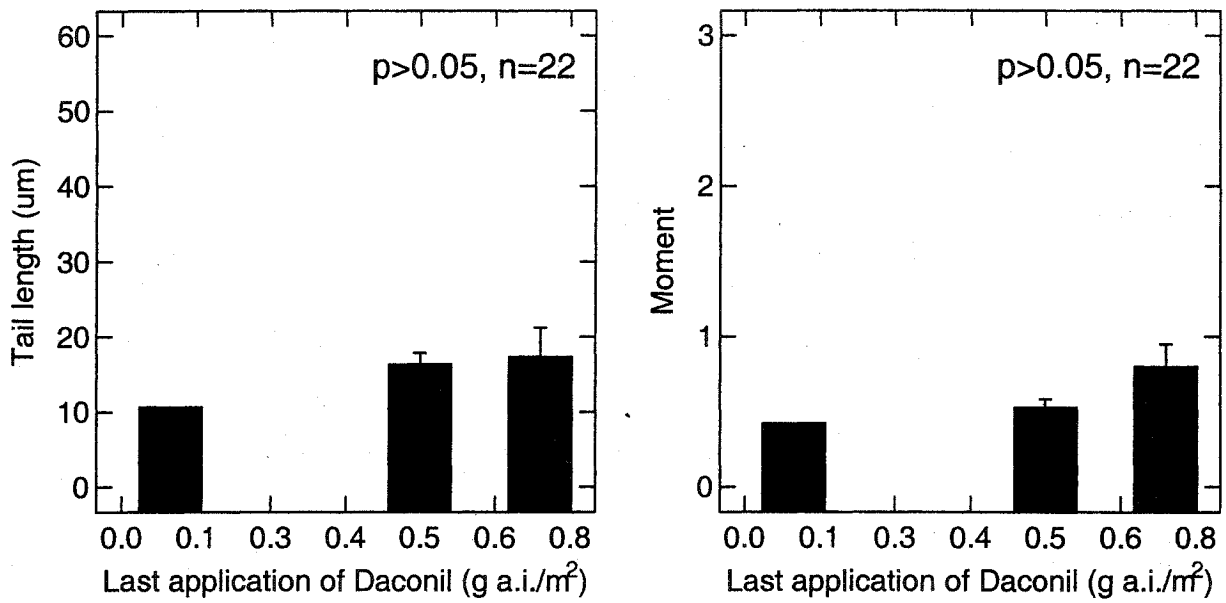


Figure 11.: Tail length and tail moment for meadow voles (*Microtus pennsylvanicus*) in relation to the last amount of Daconil® applied (g/m^2) to the capture area for a) 2001 and b) 2002. In 2002, the asterisk above the data points indicates that these points are significantly greater than the others. Data points are presented as means with standard error.

a.



b.

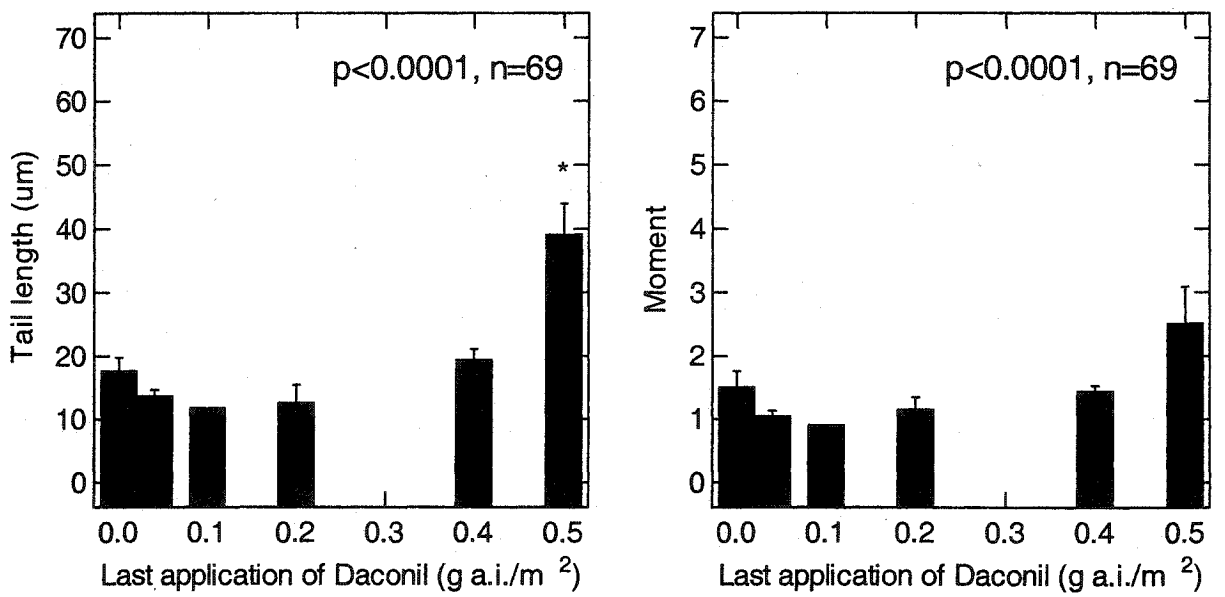


Figure 12.: Values of $FA^{C}ALL$ (composite index of fluctuating asymmetry (FA) based on the sum of absolute FA for all five traits) and $FA^{C}ITF$ (composite index based on the sum of absolute FA for ilium, tibia and femur) for meadow voles (*Microtus pennsylvanicus*) from each capture site (golf courses: A-E, reference sites: REF). Reference animals from both sites have been pooled. Values are given as means and standard error. Sample sizes at each site are placed above error bars.

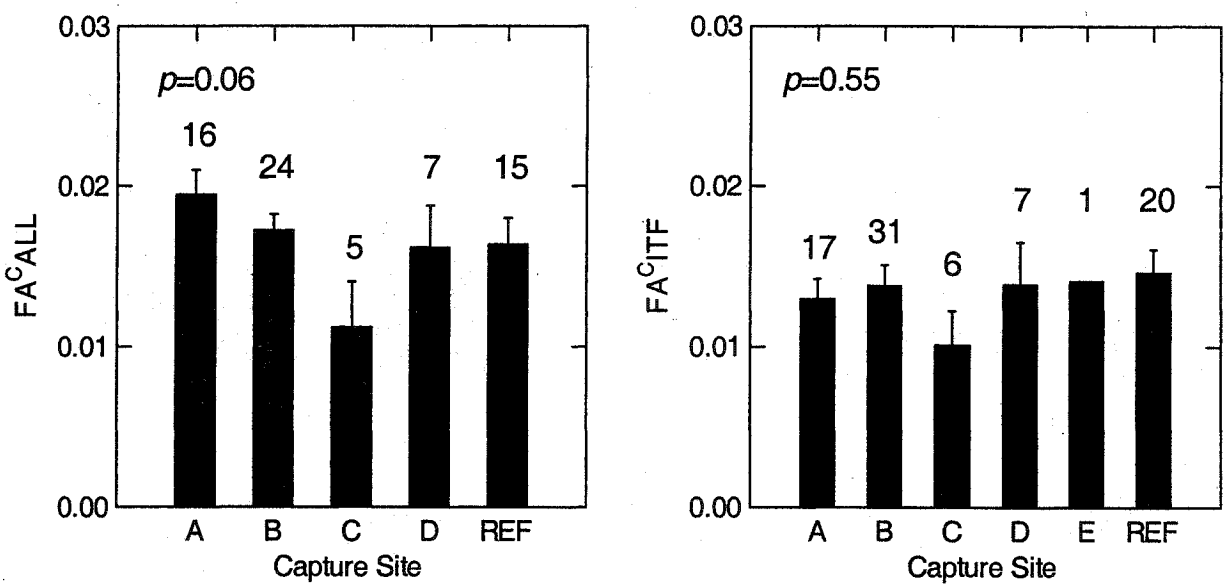


Figure 13.: Bone density values of the ilium and femur of meadow voles (*Microtus pennsylvanicus*) from each capture site (golf courses: A-E, reference sites: REF). Reference animals from both sites have been pooled. Density values are given as means and standard error, with reproductive animals and non-reproductive animals represented by black and white bars respectively. Sample sizes for ilium and femur are the same and are only represented on the graph of ilium density.

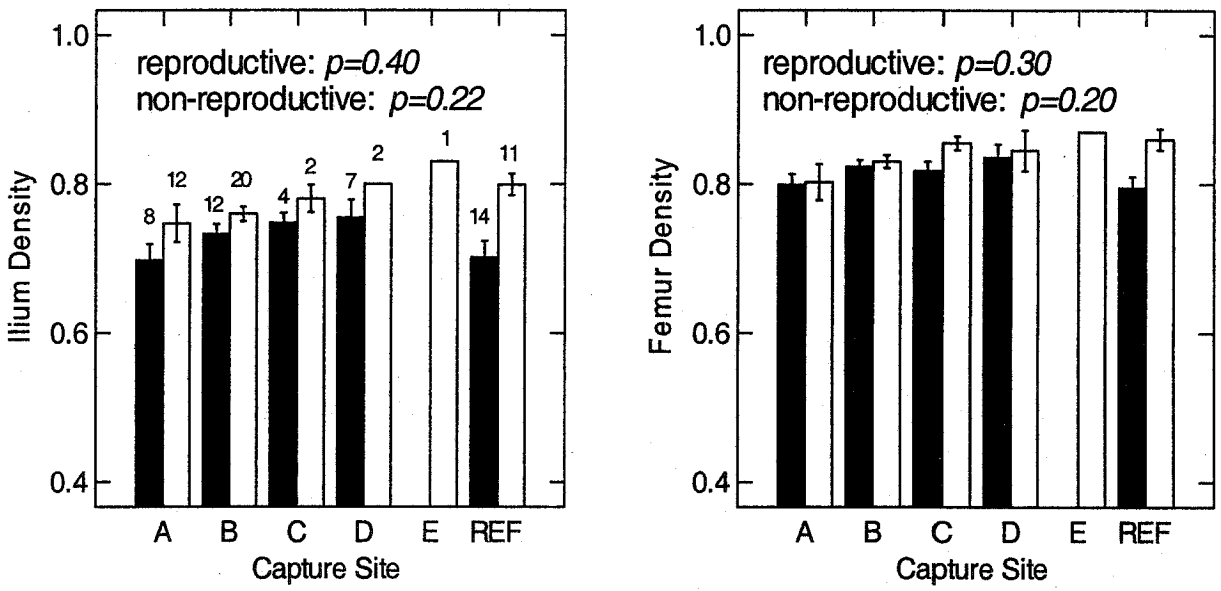


Figure 14.: Average body condition values (mass-length residuals generated from linear regression of $\log_{10}$ body mass against $\log_{10}$ body length) of meadow voles (*Microtus pennsylvanicus*) from each capture site (golf courses: A-F, reference sites: REF). Reference animals from both sites have been pooled. Values are given as means and standard error. Sample sizes at each site are placed above error bars.

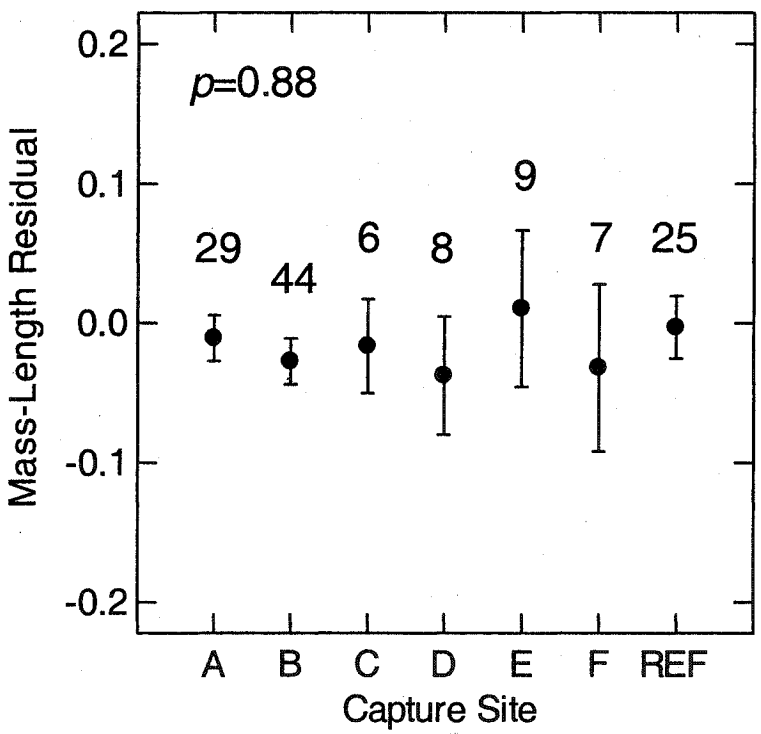
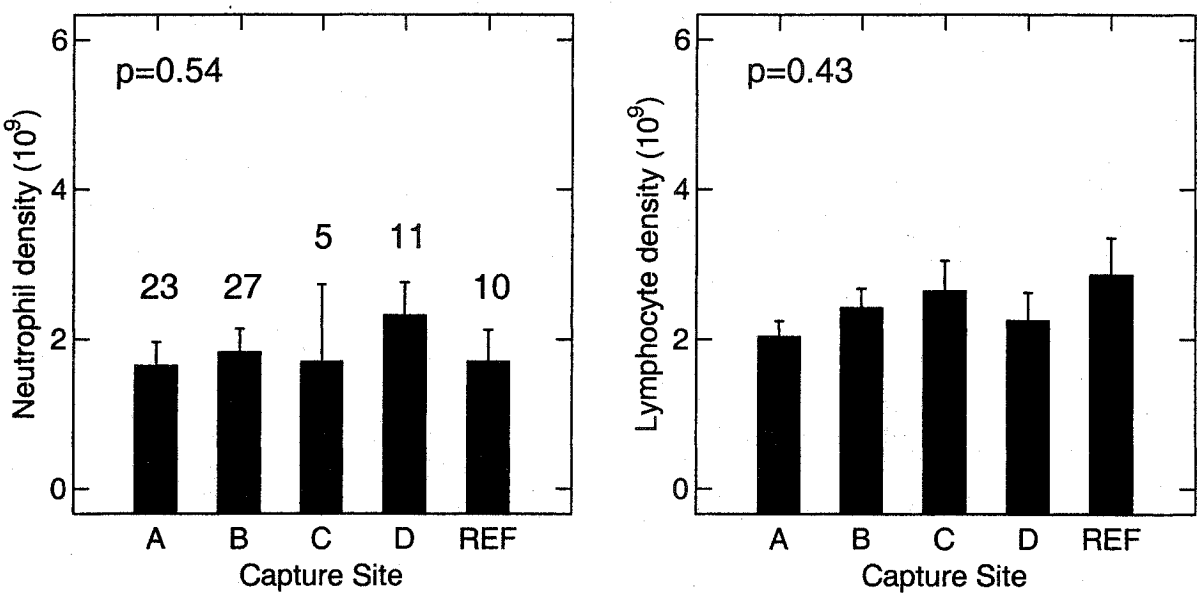


Figure 15.: Neutrophil and lymphocyte densities from meadow voles (*Microtus pennsylvanicus*) from each capture site (golf courses: A-D, reference sites: REF). Reference animals from both sites have been pooled. Values are given as means and standard error. Sample sizes for each graph are the same, and have been shown above the error bars of neutrophils only.



The following table shows the total yearly application of the most predominately used pesticides on golf courses of the Ottawa/Gatineau region. Other pesticides used during this time period are described in the text.

Table 3: Total yearly application of the most predominately used pesticides (g active ingredient/m^2) from each participating golf course from the Ottawa/Gatineau region. n/u indicates that the active ingredient was not used, spot indicates that products containing the active ingredient were applied as spot treatments throughout the course but total application rates cannot be determined.

Golf Course	Year	Size of application area (m^2)	Active ingredient (g/m^2/year)				
			Chlorothalonil	Iprodione	2,4-D	Glyphosate	Diazinon
A	2001	9,290	4.0	0.3	n/u	n/u	1.0
	2002		2.0	n/u	n/u	n/u	n/u
B	2001	13,800	0.9	n/u	spot	0.3	n/u
	2002		3.0	0.008	spot	n/u	3.0
C	2001	197,000	0.2	n/u	n/u	n/u	n/u
	2002		0.4	0.2	spot	n/u	spot

D	- 2002	 11,148- 101,171	- 2.0	- 0.3	- spot	- spot	- 0.06
E	2001 -	9290	0.9 -	n/u -	n/u -	n/u -	n/u -

Table 4.: Statistics for signed fluctuating asymmetry of humerus, ulna, ilium, femur and tibia measured on wild meadow voles (*Microtus pennsylvanicus*) from reference sites and golf courses during 2001-2003. CI: confidence intervals, SE: standard error.

Trait	*n*	Mean	95%CI+	95% CI-	SE Skewness	SE Kurtosis
Humerus	69	0.002	0.012	-0.007	0.289	0.570
Ulna	69	-0.003	0.004	-0.011	0.289	0.570
Ilium	85	0.004	0.007	0.001	0.261	0.517
Femur	85	0.000	0.007	-0.008	0.261	0.517
Tibia	84	-0.001	0.002	-0.004	0.263	0.520

Table 5.: Blood parameters from differential leukocyte counts for meadow voles (*Microtus pennsylvanicus*) captured from golf courses and reference sites in 2002. Reference animals from both sites have been pooled. Values are given as means from each site (10^9 cells/L) and standard error. WBC: white blood cells.

Golf Course	*n*	WBC	Neutrophils	Lymphocytes	Monocytes	Eosinophils	Basophils
A	23	4.4 (0.5)	1.8 (0.3)	2.3 (0.3)	0.15 (0.05)	0.06 (0.02)	0.04 (0.01)
B	27	5.2 (0.6)	2.3 (0.4)	2.6 (0.3)	0.13 (0.04)	0.08 (0.03)	0.04 (0.02)
C	5	4.6 (1.1)	1.7 (0.9)	2.6 (0.4)	0.06 (0.04)	0.14 (0.02)	0.04 (0.02)
D	11	4.5 (0.5)	2.2 (0.4)	2.2 (0.3)	0.06 (0.02)	0.03 (0.01)	0.0 (0.0)
Ref	10	5.2 (0.6)	1.7 (0.4)	3.3 (0.6)	0.07 (0.02)	0.07 (0.03)	0.05 (0.02)

CHAPTER 4:

SUMMARY OF THESIS

This thesis was comprised of two main components; 1) a detailed review of the cancer causing potential and genetic damaging genotoxic potential of the most commonly used pesticides on golf courses of the Ottawa/Gatineau region, and 2), the results of the field study conducted using meadow voles (*Microtus pennsylvanicus*) as an indicator species for a biomonitoring study.

1. CANCER CAUSING POTENTIAL OF GOLF COURSE PESTICIDES

There appears to be convincing cellular and organismal evidence to support the claim that under certain circumstances, iprodione, chlorothalonil, PMA and 2,4-D have been associated with cancer in humans and animals. Carcinogenicity from these pesticides seems to be related to genetic and non-genetic effects. At the present time, I am unaware of any ongoing studies on health outcomes of animals or people that are likely to come into contact with these common golf course pesticides through occupational contact. Indeed, golf course professionals, people who could be exposed to these individual pesticides and mixtures of these pesticides for extended periods of time, appear under represented in occupational studies dealing with the effects of long-term pesticide exposure. Aside from one study (Kross et al. 1996), where a link between cancer-related mortality and pesticide exposure was presented (but was not directly measured due to study design constraints), researchers interested in pesticides and golf courses have not written about health effects related to exposure. Rather studies have focused on pesticide runoff, the accumulation and movement of pesticide residues in soil, and contamination of ground water by pesticides. In view of the fact that there is in vitro and in vivo laboratory and epidemiological evidence suggesting that under certain circumstances commonly used golf course pesticides have

been associated with the onset of animal and human cancer, I suggest that there is a need to conduct research into the health effects of those individuals likely to be chronically exposed to these pesticides. Golf course superintendents, their staff, and the wildlife found on golf courses are likely candidates for such studies.

2. FIELD COMPONENT

Results of my field studies suggest that in general, meadow voles from golf courses did not appear to differ in their asymmetry, skeletal birth defects, blood parameters, parasite load, body condition or level of chromosomal damage when compared to voles from reference sites. Thus, overall, voles found on golf courses were no less "healthy" than animals from reference sites. However, high type II error associated with the organismal biomarkers mean I can only say with certainty that I am not able to falsify the null hypotheses that no relationship exists between FA, birth defects, haematology and parasite load and pesticide exposure. It is also possible that animals with severe health concerns had been removed from the population through selection and were not present for this study. Thus, only relatively "healthy" animals may have been captured.

At the molecular level, however, voles from golf courses did appear to experience significantly greater levels of DNA strand breaks (in white blood cells) than voles from reference sites. There was considerable intra-golf course variation in levels of DNA damage but since no one golf course had animals that all had high levels of DNA damage, there appears to be nothing intrinsic about any of the golf courses that could cause genotoxicity (e.g., golf cart emissions, chemical spills, radon gas from geological features). It is possible that this variation is simply related to varying exposure rates, but it is also

possible that variation was related to hormesis, and animals that have been previously exposed to pesticides capable of initiating DNA damage have elevated rates of DNA repair and are better able to mend strand breaks more efficiently.

DNA damage is generally the first step in the cascading events leading to cancer and eventual cellular and organismal death and the possible heritable transfer of genetic mutation. Because DNA damage in voles appears to decrease in relation to the days since their encounter with an application, with damage after 10-15 days no different than that in reference animals, the health related effects of this damage is uncertain. However, DNA damage in human sperm has been directly related to male infertility. Voles are very prolific breeders, and it is possible that during the first 10-15 days after an application, male voles may have DNA damage in their sperm that could lead to their infertility. In a species where rapid reproduction is essential, decreased fertility could have detrimental effects on their population and on the species that rely on them. This relationship is something that remains to be investigated.

APPENDIX I

Power Analysis

When mean values of endpoints were compared among capture sites, a post-hoc power analysis was conducted to determine how many animals from each capture site would be needed to correctly reject the null hypothesis that no difference among capture sites exists, based on 80% probability. Animals from each capture site were pooled together and a site mean and standard deviation (SD) generated. An analysis of variance (ANOVA) was then conducted to determine the within group variance (e.g., mean square error (MSE)). The square root of the MSE is the SD for the relationship between sites and trait (within cell SD), and this SD was used for the one-way ANOVA power analysis. For the power analysis (SYSTAT vers. 10) the mean value of the endpoint from each capture site and the within cell SD were used, and an alpha value of 0.05 and a power value of 0.80 selected in the SYSTAT power analysis window.

Power analysis: $FA^{C}ALL$ and $FA^{C}ITF$

	$FA^{C}ALL$			**$FA^{C}ITF$**		
Site	**Mean**	**SD**	**N**	**Mean**	**SD**	**n**
Golf course 1	0.118	0.061	16	0.045	0.028	17
Golf course 2	0.095	0.039	24	0.052	0.030	31
Golf course 3	0.056	0.028	5	0.030	0.014	6
Golf course 4	0.081	0.031	7	0.042	0.019	7
Reference	0.082	0.031	15	0.044	0.019	20

$FA^{C}ALL$: MSE=0.02, SD=0.141, therefore, 113 voles per site are required for a power of 80% (i.e, in order to have an 80% probability of correctly rejecting the null hypothesis)

FA^{C}ITF: MSE=0.01, SD=0.10, therefore, 469 voles per site are required for a power of 80% (i.e, in order to have an 80% probability of correctly rejecting the null hypothesis).

Power analysis: Body condition

	MLR		
Site	**Mean**	**SD**	**N**
Golf course A	0.022	0.124	29
Golf course B	0.002	0.137	44
Golf course C	-0.017	0.074	6
Golf course D	-0.063	0.112	8
Golf course E	0.014	0.212	9
Golf course F	-0.010	0.251	7
Reference	-0.006	0.140	25

MSE=0.021, SD=0.145, therefore, 64 voles per site are required for a power of 80% (i.e, in order to have an 80% probability of correctly rejecting the null hypothesis).

Power Analysis: Micronucleated polychromatic erythrocyte (MN-PCE)

	MN-PCE		
Site	**Mean**	**SD**	**N**
Golf course 1	0.120	0.124	20
Golf course 2	0.080	0.118	25
Golf course 3	0.100	0.061	5

Golf course 4	0.141	0.132	11
Reference	0.095	0.093	10

MSE=0.014, SD=0.118, therefore, 75 voles per site are required for a power of 80% (i.e, in order to have an 80% probability of correctly rejecting the null hypothesis).

Power Analysis: Absolute white blood cell levels (x 10^9 cells)

	WBC		
Site	**Mean**	**SD**	**N**
Golf course A	4.357	2.565	23
Golf course B	5.244	2.849	27
Golf course C	4.560	2.509	5
Golf course D	4.482	1.575	11
Reference	5.230	2.008	10

MSE=6.227, SD=2.495, therefore, 103 voles per site are required for a power of 80% (i.e, in order to have an 80% probability of correctly rejecting the null hypothesis).

APPENDIX II

ACTUAL CAPTURE DATES AND CAPTURE SITES OF MEADOW VOLES USED IN THE FIELD STUDY

Date of capture	Course	Capture Site	Vole #
July 2, 2001	B	3tee+fwy	1
July 2, 2001	B	3tee+fwy	2
July 2, 2001	B	3tee+fwy	3
July 2, 2001	B	3tee+fwy	4
July 2, 2001	B	3tee+fwy	5
July 9, 2001	A	4fwy	6
July 9, 2001	A	4fwy	7
July 9, 2001	A	4fwy	8
July 9, 2001	A	2green	9
July 9, 2001	A	2green	10
July 12, 2001	E	3W-green	11
July 16, 2001	E	17-green	12
July 20, 2001	F	18tee	13
July 20, 2001	F	18tee	14
September 10, 2001	E	4green	15
September 11, 2001	E	4green	16
September 12, 2001	E	4green	17
September 12, 2001	E	3green	18
September 13, 2001	E	3green	19
September 13, 2001	E	3green	20
September 17, 2001	B	3tee+fwy	21
September 17, 2001	B	3tee+fwy	22
September 17, 2001	B	3tee+fwy	23
September 18, 2001	B	3tee+fwy	24
September 18, 2001	B	3tee+fwy	25
September 18, 2001	B	3tee+fwy	26
September 19, 2001	B	3tee+fwy	27
September 19, 2001	B	3tee+fwy	28
September 19, 2001	B	3tee+fwy	29
September 20, 2001	F	18tee	30
September 24, 2001	F	18W-fwy	31
September 24, 2001	F	18W-fwy	32
September 25, 2001	F	18tee	33
September 25, 2001	F	18W-fwy	34
September 25, 2001	F	18W-fwy	35
September 25, 2001	F	18W-fwy	36
September 26, 2001	B	3tee+fwy	37
September 26, 2001	B	3tee+fwy	38
September 26, 2001	B	3tee+fwy	39
September 26, 2001	B	3tee+fwy	40
September 27, 2001	B	3tee+fwy	41

September 27, 2001	B	3tee+fwy	42
September 27, 2001	B	3tee+fwy	43
September 28, 2001	A	4fwy	44
September 28, 2001	A	4fwy	45
September 28, 2001	A	4fwy	46
September 28, 2001	A	4fwy	47
September 28, 2001	A	4fwy	48
September 28, 2001	A	4fwy	49
May 30, 2002	A	7tee	50
May 31, 2002	A	2green	51
May 31, 2002	A	4fwy	52
June 10, 2002	B	3tee+fwy	53
June 13, 2002	B	3tee+fwy	54
June 14, 2002	B	3tee+fwy	55
June 18, 2002	A	2green	56
June 18, 2002	A	4fwy	57
June 18, 2002	A	4fwy	58
June 18, 2002	A	4fwy	59
June 19, 2002	A	2green	60
June 19, 2002	A	2green	61
June 19, 2002	A	4fwy	62
June 19, 2002	A	4fwy	63
June 20, 2002	A	7tee	64
June 20, 2002	A	7tee	65
June 20, 2002	A	4fwy	66
June 20, 2002	A	4fwy	67
June 20, 2002	A	4fwy	68
June 20, 2002	A	4fwy	69
June 20, 2002	A	2green	70
June 24, 2002	C	4tee	71
July 2, 2002	C	4tee	72
July 4, 2002	C	6tee	73
July 5, 2002	B	4tee	74
July 5, 2002	B	4tee	75
July 5, 2002	B	4tee	76
July 5, 2002	B	4tee	77
July 5, 2002	B	4tee	78
July 5, 2002	B	4tee	79
July 5, 2002	B	4tee	80
July 5, 2002	B	4tee	81
July 5, 2002	B	4tee	82
July 8, 2002	B	3tee+fwy	83
July 8, 2002	B	3tee+fwy	84
July 8, 2002	B	4tee	85

July 9, 2002	B	3tee+fwy	86
July 9, 2002	B	4tee	87
July 10, 2002	B	3tee+fwy	88
July 11, 2002	B	3tee+fwy	89
July 18, 2002	D	14fwy	90
July 22, 2002	D	14tee	91
July 22, 2002	D	14tee	92
July 22, 2002	D	14tee	93
July 22, 2002	F	14tee	94
July 23, 2002	F	14tee	95
July 24, 2002	F	14tee	96
July 25, 2002	C	6tee	97
July 29, 2002	C	4tee	98
July 30, 2002	C	4tee	99
July 31, 2002	A	2green	100
July 31, 2002	A	4fwy	101
July 31, 2002	A	4fwy	102
July 31, 2002	A	4fwy	103
August 1, 2002	A	4fwy	104
August 8, 2002	Reference Site 1	Meadow	105
August 9, 2002	Reference Site 1	Meadow	106
August 19, 2002	B	4tee	107
August 19, 2002	B	3tee+fwy	108
August 21, 2002	B	4tee	109
August 21, 2002	B	4tee	110
August 22, 2002	B	4tee	111
August 22, 2002	B	4tee	112
August 26, 2002	D	14tee	113
August 27, 2002	D	14tee	114
August 27, 2002	D	14tee	115
September 9, 2002	Reference Site 2	Meadow	116
September 9, 2002	Reference Site 2	Meadow	117
September 9, 2002	Reference Site 2	Meadow	118
September 10, 2002	Reference Site 2	Meadow	119
September 10, 2002	Reference Site 2	Meadow	120
September 10, 2002	Reference Site 2	Meadow	121
September 12, 2002	Reference Site 2	Meadow	122
September 12, 2002	Reference Site 2	Meadow	123
June 8, 2003	Reference Site 2	Meadow	124
June 8, 2003	Reference Site 2	Meadow	125
June 8, 2003	Reference Site 2	Meadow	126
June 8, 2003	Reference Site 2	Meadow	127
June 8, 2003	Reference Site 2	Meadow	128
June 8, 2003	Reference Site 2	Meadow	129

June 10, 2003	Reference Site 2	Meadow	130
June 10, 2003	Reference Site 2	Meadow	131
June 11, 2003	Reference Site 2	Meadow	132
June 11, 2003	Reference Site 2	Meadow	133
June 11, 2003	Reference Site 2	Meadow	134
June 11, 2003	Reference Site 2	Meadow	135
June 11, 2003	Reference Site 2	Meadow	136
June 12, 2003	Reference Site 2	Meadow	137
June 12, 2003	Reference Site 2	Meadow	138
June 12, 2003	Reference Site 2	Meadow	139
June 12, 2003	Reference Site 2	Meadow	140
June 12, 2003	Reference Site 2	Meadow	141
June 16, 2003	Reference Site 1	Meadow	142

APPENDIX III

WEATHER CONDITIONS DURING CAPTURE PERIOD

Date	Max Temp(°C)	Min Temp(°C)	Mean Temp (°C)	Max Hum(%)	Rainfall (mm)	Min Hum (%)
1-Jul-01	23	8.5	15.8	95	2.8	47
2-Jul-01	20	8.2	14.1	88	0	44
3-Jul-01	19.3	12.1	15.7	89	0.4	56
4-Jul-01	24.4	13	18.7	97	17.2	67
5-Jul-01	22.7	9.9	16.3	88	0	33
6-Jul-01	18.7	8.3	13.5	94	0	54
7-Jul-01	25.8	12.8	19.3	87	0.4	41
8-Jul-01	27	16.6	21.8	94	0	47
9-Jul-01	29.6	15.8	22.7	100	6.6	39
10-Jul-01	25	15.6	20.3	96	2.6	50
11-Jul-01	21.1	13.1	17.1	100	5.8	55
12-Jul-01	19.4	10.8	15.1	95	0.8	70
13-Jul-01	21.6	14.7	18.2	85	0	64
14-Jul-01	22	14.6	18.3	91	0	66
15-Jul-01	24.7	13.3	19	97	0	0
16-Jul-01	27.4	13.8	20.6	93	0	37
17-Jul-01	27.3	15.6	21.5	87	0	36
18-Jul-01	27.3	14.7	21	90	0	46
19-Jul-01	28.5	14.9	21.7	94	0	42
20-Jul-01	30.4	15.3	22.9	87	0	42
21-Jul-01	30	16.8	23.4	87	0	52
22-Jul-01	32	19.1	25.6	86	0	42
23-Jul-01	33.8	20	26.9	94	2	50
24-Jul-01	34.1	20.9	27.5	89	0	38
25-Jul-01	26.6	15.6	21.1	83	0	44
26-Jul-01	21.7	11.3	16.5	71	0	31
27-Jul-01	24	8.6	16.3	88	0	31
28-Jul-01	25.4	11.1	18.3	84	0	32
29-Jul-01	28.4	14.5	21.5	81	0	35
30-Jul-01	25.5	17.1	21.3	96	0	58
31-Jul-01	30.4	15.6	23	99	0	31
1-Aug-01	33.1	16.4	24.8	77	0	28
2-Aug-01	30	17.4	23.7	100	23.8	40
3-Aug-01	30.3	20.1	25.2	100	0	46
4-Aug-01	29.6	17.7	23.7	74	0	33
5-Aug-01	34.1	17.3	25.7	84	0	32
6-Aug-01	35.1	18.6	26.9	72	0	36
7-Aug-01	34.2	22.4	28.3	81	0	43
8-Aug-01	25.8	19.1	27.5	95	0	30
9-Aug-01	26.9	22.5	29.7	79	0	37

10-Aug-01	28.4	17.1	22.8	73	0	35
11-Aug-01	26.5	14.6	20.6	80	0	39
12-Aug-01	29.6	14.9	22.3	81	0	34
13-Aug-01	25.3	13.7	19.5	88	0.2	50
14-Aug-01	26.6	11.8	19.2	97	0	30
15-Aug-01	29.5	11.4	20.5	92	0	27
16-Aug-01	30.4	13.6	22	83	0	37
17-Aug-01	28.3	17.2	22.8	99	10	47
18-Aug-01	27.3	17.4	22.4	92	0	50
19-Aug-01	25.8	16	20.9	91	0.2	55
20-Aug-01	25.4	16.9	21.2	100	5.2	71
21-Aug-01	25.5	17	21.3	100	0	59
22-Aug-01	29.7	15.6	22.7	94	0	35
23-Aug-01	26.1	16	21.1	91	0	61
24-Aug-01	24	12.2	18.1	76	0	30
25-Aug-01	26.8	9	17.9	92	0	26
26-Aug-01	27.8	14.5	21.2	99	19.2	62
27-Aug-01	27.3	14.3	20.8	98	0	40
28-Aug-01	21.2	12.4	16.8	100	6.4	65
29-Aug-01	19.8	9.9	14.9	90	0	50
30-Aug-01	26.5	8.9	17.7	92	0	36
31-Aug-01	24.9	12.1	18.5	93	3.6	47
1-Sep-01	17	8.5	12.8	85	0	53
2-Sep-01	19.5	5.7	12.6	100	0	42
3-Sep-01	27.3	13.3	20.3	96	0.8	42
4-Sep-01	21.9	9.3	15.6	98	2.4	65
5-Sep-01	20	7.7	13.9	88	0	43
6-Sep-01	25.7	7.6	16.7	94	0	39
7-Sep-01	30.4	11.6	21	96	0	39
8-Sep-01	32.4	20.5	26.5	85	0	48
9-Sep-01	31.9	21.2	26.6	80	0	48
10-Sep-01	26	14.9	20.5	84	0.2	44
11-Sep-01	22.3	12	17.2	88	0	33
12-Sep-01	24	8.6	16.3	99	4.8	48
13-Sep-01	17.5	7.2	12.4	98	2	53
14-Sep-01	17.4	5.8	11.6	93	0	46
15-Sep-01	19.9	4.4	12.2	100	0	43
16-Sep-01	22.1	6.3	14.2	98	0	42
17-Sep-01	24	7.8	15.9	100	0	44
18-Sep-01	19.5	12.7	16.1	97	0	69
19-Sep-01	24.1	10.4	17.3	100	0.4	48
20-Sep-01	19	15	17	99	11.8	89
21-Sep-01	23.7	14.3	19	99	2.2	55
22-Sep-01	21.8	14.2	18	100	4	63

23-Sep-01	22.2	12	17.1	100	0	53
24-Sep-01	24	11.1	17.6	99	31.6	64
25-Sep-01	17.2	10.3	13.8	100	14.8	84
26-Sep-01	15.3	5.9	10.6	100	0	61
27-Sep-01	14.8	4.8	9.8	100	0.4	63
28-Sep-01	14.1	7.4	10.8	100	3	72
29-Sep-01	17.6	4.8	11.2	97	0	50
30-Sep-01	18.3	4.5	11.4	97	0	38
20-May-02	11.8	2	6.9	83	50	0.4
21-May-02	15.2	2.7	9	85	0	30
22-May-02	20.2	4	12.1	68	0	27
23-May-02	22.3	6.1	14.2	71	0	29
24-May-02	20.7	4	12.4	68	0	37
25-May-02	16.8	1	8.9	71	0	33
26-May-02	19.1	8.3	13.7	93	0.4	36
27-May-02	20.8	8	14.4	81	0	53
28-May-02	26	13	19.5	85	0.2	42
29-May-02	28.1	11.8	20	84	0	47
30-May-02	24.5	16.6	20.6	93	2.2	70
31-May-02	23.4	13.4	18.4	90	13.6	64
1-Jun-02	24.9	11.1	18	87	0	30
2-Jun-02	16.6	1.6	12.1	63	0	36
3-Jun-02	17	3.8	10.4	74	0	36
4-Jun-02	20.9	5	13	94	0.6	34
5-Jun-02	18.9	10.4	14.7	96	7.8	82
6-Jun-02	18.4	9.7	14.1	94	0	35
7-Jun-02	21.7	7.8	14.8	67	0	36
8-Jun-02	19.8	11.1	15.5	76	0	58
9-Jun-02	24.7	13.8	19.3	87	0	43
10-Jun-02	20.3	10.5	15.4	84	1	44
11-Jun-02	13.8	10.8	12.3	95	52	79
12-Jun-02	20.1	11.3	15.7	95	11.6	67
13-Jun-02	24.2	10.5	17.4	97	0	40
14-Jun-02	21.1	12.9	17	94	1.2	57
15-Jun-02	14.2	10.3	12.3	95	32.2	91
16-Jun-02	13.4	9.9	11.7	97	18.4	92
17-Jun-02	16.7	11.7	14.2	97	13	77
18-Jun-02	21.7	12.1	16.9	97	0	58
19-Jun-02	25.1	10	17.6	98	0	44
20-Jun-02	28.7	13.4	21.1	89	0	40
21-Jun-02	29.7	16.7	23.2	79	0	53
22-Jun-02	25.1	18.1	21.6	82	0	55
23-Jun-02	30	16.4	23.2	96	12	59
24-Jun-02	24.4	10	17.2	79	0	41

25-Jun-02	26.1	14.5	20.3	87	0	49
26-Jun-02	27.5	15.3	21.4	100	18	68
27-Jun-02	26.1	17.7	21.9	96	57	72
28-Jun-02	27.3	15.5	21.4	91	0	44
29-Jun-02	27.5	13.8	20.7	91	0	36
30-Jun-02	29.8	17	23.4	85	0	55
1-Jul-02	32.8	20.1	26.5	83	0	57
2-Jul-02	34.5	23.3	28.9	89	0	54
3-Jul-02	33.8	22.2	28	86	0	49
4-Jul-02	30.3	15.7	23	81	0	34
5-Jul-02	22.2	15.3	18.8	82	0	53
6-Jul-02	28	15.1	21.6	78	0	32
7-Jul-02	30.3	15.3	22.8	80	19.8	37
8-Jul-02	27	18.3	22.7	94	2	61
9-Jul-02	27.4	14.6	21	94	0	47
10-Jul-02	20.8	11	15.9	77	0	39
11-Jul-02	22.3	9.1	15.7	83	0	37
12-Jul-02	26.5	11.1	18.8	89	0	43
13-Jul-02	28.1	13.6	20.9	82	0	51
14-Jul-02	29.6	15.9	22.8	85	0	40
15-Jul-02	25.8	15.7	20.8	79	1	43
16-Jul-02	25.4	12.6	19	83	0	46
17-Jul-02	29.3	16.4	22.9	91	2.4	53
18-Jul-02	23.4	15.1	19.3	89	0	50
19-Jul-02	25.3	13.5	19.4	91	0	46
20-Jul-02	26.1	14.3	20.2	73	0	37
21-Jul-02	30.2	14.9	22.6	80	0	39
22-Jul-02	33.1	21.2	27.2	88	0.2	56
23-Jul-02	22.6	13.4	18	92	7.8	43
24-Jul-02	23.6	11.1	17.4	83	0	45
25-Jul-02	24.7	13.2	19	82	0	41
26-Jul-02	19.5	15.9	17.7	94	13.8	72
27-Jul-02	27.6	15.3	21.5	95	0	59
28-Jul-02	28.4	17.4	22.9	91	0	63
29-Jul-02	30.7	21.6	26.2	90	0.4	54
30-Jul-02	30.6	21.1	25.9	93	0.4	44
31-Jul-02	32.4	20.2	26.3	80	0	40
1-Aug-02	32.3	19.8	26.1	89	0	44
2-Aug-02	29.8	20.5	25.2	93	6	51
3-Aug-02	29.6	16.8	23.2	89	0	38
4-Aug-02	27.6	16.3	22	88	0.2	54
5-Aug-02	28.9	14.2	21.6	90	0	40
6-Aug-02	20.8	12.4	16.6	73	0	52
7-Aug-02	25	13.2	19.1	76	0	44

8-Aug-02	26.2	12.1	19.2	81	0	34
9-Aug-02	27.9	12.2	20.1	90	0	38
10-Aug-02	31	16.6	23.8	73	0	33
11-Aug-02	32.8	15.1	24	85	0	34
12-Aug-02	34	18.2	26.1	84	0	36
13-Aug-02	32.7	19.7	26.2	89	0	47
14-Aug-02	35.2	19.1	27.2	87	0	38
15-Aug-02	28.7	21.6	25.2	91	2.9	65
16-Aug-02	31.9	18.7	25.3	94	1.2	54
17-Aug-02	30.4	15.4	22.9	88	0	38
18-Aug-02	32.4	17.4	24.9	86	0	32
19-Aug-02	23.3	14	18.7	93	1.8	54
20-Aug-02	26	13.4	19.7	96	0	36
21-Aug-02	27.6	10.7	19.2	88	0	38
22-Aug-02	18.8	12.7	15.8	95	27.6	66
23-Aug-02	23.3	11.8	17.6	93	0	39
24-Aug-02	23.6	12.5	18.1	94	0	61
25-Aug-02	23.3	13.1	18.2	96	0	49
26-Aug-02	28.3	12	20.2	91	0	50
27-Aug-02	21.5	11.4	16.5	75	0	42
28-Aug-02	23	10.4	16.7	82	0	43
29-Aug-02	24.8	11.9	18.4	86	0	51
30-Aug-02	27.8	11	19.4	95	0	49
31-Aug-02	26.6	14	20.3	82	0	53
1-Sep-02	26.6	14	20.3	92	0	55
2-Sep-02	25.3	11.2	18.3	91	0	57
3-Sep-02	28.5	15	21.8	100	0	6
4-Sep-02	25.6	14.1	19.9	83	0	48
5-Sep-02	21.5	10.7	16.1	74	0	39
6-Sep-02	25.1	16.6	16.6	90	0	38
7-Sep-02	31.3	13.2	22.3	91	0	39
8-Sep-02	34.5	16.1	25.3	76	0	37
9-Sep-02	35.1	18.2	26.7	80	0	31
10-Sep-02	33.5	17.7	25.6	93	19.2	40
11-Sep-02	19.6	8.9	14.3	91	9.2	45
12-Sep-02	20.9	5.8	13.4	89	0	43
8-Jun-03	29.3	15.1	22.2	92	0	68
9-Jun-03	22.6	13.2	17.9	96	2	54
10-Jun-03	22.9	9.7	16.3	94	0	34
11-Jun-03	23.4	13.4	18.4	97	7	65
12-Jun-03	24.7	9.7	17.2	97	0	32
13-Jun-03	16.8	14.2	15.5	97	6	60
14-Jun-03	21.3	13.9	17.6	97	1	68
15-Jun-03	24.2	13.8	19	97	0	28

16-Jun-03	25.1	8.1	17.9	89	0	34

APPENDIX IV

FLUCTUATING ASYMMETRY IN BOBWHITE QUAIL CHICKS (*COLINUS VIRGINIANUS*) DOES NOT FOLLOW A PREDICTABLE DOSE-RESPONSE RELATIONSHIP FOLLOWING MATERNAL EXPOSURE TO FOUR DIFFERENT HERBICIDES

INTRODUCTION

As mentioned in Chapter 1, an attractive feature of using fluctuating asymmetry (FA) as a biomarker of anthropogenic stress is its relative ease in measurement. As long as researchers have callipers to measure bilaterally paired traits, FA data can easily be generated. Recently Leung, Knopper and Mineau (2003) examined FA's potential as a biomarker by evaluating it against nine criteria that have been proposed for ideal biomarkers of anthropogenic stress (e.g., biological relevance and robustness, ease, permanence of response, etc.). We indicated that most studies that have investigated the relationship between FA and anthropogenic stressors in ecotoxicology have done so by measuring organisms from polluted ecosystems and comparing them to organisms at reference sites, with the *a priori* prediction that unstressed organisms will have a more perfect bilaterally symmetry. What we suggested is what has received little attention in the FA literature is whether or not FA follows a predictable dose-response relationship with stress, a key criterion of a useful biomarker (Mayer et al., 1992, Clarke, 1993, Mineau, 1998). Moreover, we found studies comparing the sensitivity of FA to other biomarkers were also generally lacking. Four notable exceptions stand out, especially since these papers come to different conclusions about the efficacy of FA as a biomarker. Valentine and Soulé (1973) reported a clear dose-response relationship with FA in pectoral fin rays of grunion fry exposed to increasing concentrations of p-p'-DDT. Mpho et al., (2001) reported that FA in wings of male mosquitoes, but not females, increased in relation to increasing exposure to the insecticide temephos. Leamy et al., (1999) found that offspring from female mice exposed to two different concentrations (0.01 and 10 μg/g body weight)

of the insecticide methoxychlor during pregnancy did not have FA any different from that of control pups. Similarly, Polak et al., (2002) found that FA from flies reared at sub-lethal concentrations of sodium arsenite did not differ from that of controls.

Considering that FA was one of the biomarkers used in this thesis as a marker of pesticide stress, I undertook this side study to determine how FA responds to pesticide stress under laboratory situations, and compared FA to life history endpoints used in the avain reproductive test. Prior to becoming registered and commercially available, most newly developed pesticides must undergo an avian reproductive test. The testing procedure entails exposing parent birds to a pesticide in varying concentrations and then tracking markers of reproductive toxicity in eggs and chicks exposed to the pesticide *in ovo*. Because these markers are measured in relation to dose and duration of pesticide exposure, these chicks are excellent models to test hypotheses about FA and pesticide stress. Accordingly, by using chicks from the currently mandated avian reproductive test we tested whether there was a relationship between FA and dose or duration of exposure of their parents to one of four different herbicides, and if FA appeared to be a more sensitive marker of stress compared to standard reproductive endpoints measured from this test (e.g., number of eggs laid, eggshell thickness, hatching success, chick survival, etc.).

MATERIALS AND METHODS

Chicks (male and female) from parent birds (Bobwhite quail, *Colinus virginianus*) exposed to one of four different herbicides were used for this study. The identity of the herbicides was withheld from us in order to respect the proprietary nature of the test results.

Parent birds were maintained at a wildlife testing facility and divided into four groups: a control group and three dose groups (low, medium, high). Birds from each dose group were given the herbicide mixed into their feed for ten weeks pre-lay. Birds from the high dose group were given a dose 2.0 or 2.5 times (depending on the compound) greater than the medium dose group, which was 2.0 or 2.5 times greater than the low dose group. Females were induced to lay eggs for a period of ten weeks by photoperiod manipulation and the number of eggs laid each week was counted. The number of cracked eggs (non-viable) laid was noted, and eggs without cracks were placed in an incubator until hatching. The number of eggs that hatched and the number of chicks that survived until 14-days of age was recorded. When chicks were 14-days old they were euthanized by exposure to CO_2 and shipped frozen to our laboratory for measurement of FA. Chicks arrived blindly labelled so no knowledge of their dosing or duration of exposure was known prior to measuring FA.

In the first, second, third, and fourth herbicide study, 212 (71 control, 141 dose), 642 (133 control, 509 dose), 441 (111 control, 330 dose) and 295 (110 control, 185 dose) chicks were measured, respectively. The occurrence of FA in these chicks was determined by measuring ulna, tarsus and central toe lengths, from the right and left side of each individual. Canthus length and the mass of primary feathers 1-5 were also originally chosen as potential traits, but the measurement error (ME) associated with these traits was very high (>15%). Consequently these traits were deemed poor candidates for the study and removed from further analysis. Measurements of bones were taken using digital callipers (Mitutoyo Absolute Digimatic Callipers, ±0.01mm) connected to a desktop computer and measurement data was automatically entered onto a spreadsheet program.

The ulna was dissected from the forearm and measured from the olecranon process to the distal head. The tarsus was externally measured from the tibio-tarsal joint to the lower scute, with the tarsus parallel to the floor and the foot bent downwards. The central toe was externally measured from the tarsal-toe joint to the base of the toenail, with the foot flat and the tarsus perpendicular to the ground. Repeated measurements of these traits were taken on the same day without the measurer having knowledge of the previous measurement value. All measurements were taken by LDK to avoid ME associated with multiple measurers (Yezerinac et al., 1992). For the first herbicide studied, FA was only measured in chicks from weeks 8 and 9 (week 1 being the first week following induction of laying) and only from control, medium and high dose groups. Chicks from the low dose group and those from earlier weeks of exposure were not available for this study. For the fourth herbicide tested, FA was only measured in chicks from control, low and medium dose groups. The highest concentration proved to be toxic to parent birds within the first few weeks of exposure and this group was abandoned from further testing. FA was measured in chicks from all four groups in the second and third herbicides tested.

Statistical analysis

If the difference between repeated measurements for each trait was ± 3 S.D. from the mean of that trait (using all birds combined within each herbicide study), then the measurement was considered an outlier. We believe that these values are not indicative of true measurement error, especially since they were so far removed from the average difference between repeated measurements, and any bird that had an outlier associated with it was removed from subsequent analyses. To calculate the ME associated with each trait,

replicate right and left side measurements for each bird (control and dose birds combined from each week of exposure) were entered in a two-way mixed model ANOVA, with "side" as the fixed variable and "individual" as the random variable (Palmer and Strobeck, 1986, Yezerinac, 1992, Swaddle et al., 1994). ME was calculated using the following equation:

[1] $\%ME = [s^2_{within} / (s^2_{within} + s^2_{among})] \times 100$

where $s^2_{among} = MS_{among} - MS_{within} / m$, and where MS_{among} and MS_{within} are the mean square deviations among and within individuals respectively, and m is the number of repeated measurements (Yezerinac et al., 1992).

FA associated with each trait was calculated as the mean right minus mean left measurement. The allometric relationship between FA and trait size was determined using Pearson correlations. Tests for skewness and kurtosis were conducted to determine the two statistical properties of FA: right minus left measurements must be approximately normally distributed and centred around a mean of zero (Palmer and Strobeck, 1992). Normal distributions are characterised by non-significant values of skewness (e.g., skewness / SE skewness (SSE) < 1.96, > -1.96) and kurtosis (e.g., kurtosis / SE kurtosis (KSE) < 1.96, > -1.96) (Sokal and Rohlf, 1981).

The occurrence of FA is more reliably detected when analyses are based on the combination of multiple traits rather than individual traits alone (Palmer and Strobeck, 1986, Leary and Allendorf 1989, Leung et al., 2000, Palmer and Strobeck, 2003). Accordingly, we calculated a composite index of FA (FA^C), defined as the sum of absolute FA from all three traits corrected by trait size (i.e., FA^C = [abs (R-L ulna)/(R+L ulna/2)] +

[abs (R-L tarsus)/(R+L tarsus/2)] + [abs (R-L toe)/(R+L toe/2)]). Because the three traits differed in their average length (i.e., tarsus > toe > ulna), FA was trait size corrected in this way to ensure that no one trait could bias the composite index do to its size. Levene's test was used to statistically test for changes in FA^C in relation to dose and duration (i.e., week of exposure) of herbicide exposure (see Palmer and Strobeck, 2003, for a detailed description of the applicability of Levene's test using absolute FA data). All statistics were conducted using Systat vers.10 for Windows.

RESULTS

Signed differences between sides for all traits measured in all four herbicide groups sometimes exhibited significant skewness and kurtosis (e.g., SSE and SEK > 1.96 or < -1.96) in both control and dosed birds, and sometimes had means that were significantly ($p<0.05$) different than zero. Inspection of the normal probability plots for each trait illustrated that departures from normality were generally very slight and for those traits with means different from zero, the 95% C.I. about the means were always extremely close to overlapping zero. Even though the distribution of these traits was at times statistically different than normal, we felt that it was not biologically relevant and indicative of deviations from ideal FA. In chicks from all four studies, %ME was low and ranged from 0.67% to 5.3%, depending on which trait was measured. ME of the ulna was lowest and that of the toe was highest. FA in the ulna, tarsus and toe from all unknown herbicide groups were not significantly related to mean trait size ($P>0.05$).

For the first herbicide, FA^C was significantly related to week of exposure ($p=0.03$) and dose ($p=0.04$) of herbicide given. However, there was no consistent dose-response relationship between weeks of exposure and dose level. In week 8, FA^C from control chicks was lower than chicks from the medium and high dose groups, but in week 9, FA^C from control chicks was greater than chicks in the medium and high dose groups (Figure 1). For the second and third herbicides testes, FA^C was not significantly related to dose or week of exposure (p values ranging from 0.14 to 0.74), and again no consistent dose-response or duration of response relationship was apparent (Figure 1). For the fourth herbicide, FA^C was not significantly related to week of exposure ($p=0.37$) but was marginally related to dose ($p=0.05$) of herbicide given. However, as in the previous cases, no consistent dose-response relationship was apparent (Figure 1). Post-hoc analysis revealed that FA from individual traits did not consistently follow a dose-response or week of exposure response in any of the four herbicide studies. Thus the use of a composite index did not unknowingly mask potential individual trait FA relations.

No significant changes in reproductive endpoints were observed regardless of the dose or duration of exposure of parent birds to herbicides one, two and three. Exposure to the fourth herbicide resulted in marked reproductive toxicity in relation to dose. The precise week of the onset of these effects could not be ascertained due to the study design. The number of eggs laid and their viability, number of hatching chicks, chick survival to 14-days of age and chick mass at 14-days of age, all decreased in a dose-dependent fashion. At the lowest dose, only body mass at 14-days of age was significantly reduced ($p<0.01$) relative to controls, but the other endpoints showed a decreasing trend. Though not significant, these decreasing trends were considered biologically relevant. At the medium

dose, all endpoints, except egg viability, were significantly reduced ($p<0.01$) relative to controls.

DISCUSSION

The use of FA as a measure of developmental stability has frequently been used as a biomarker of anthropogenic stress in a wide range of organisms. For instance, greater FA than would be expected in animals from reference sites has been found in populations of common shrew (*Sorex araneus*) exposed to heavy metal pollution (Pankakoski et al., 1992), in grey seals (*Halichoerus grypus*) and ringed seals (*Pusa hispida*) exposed to DDT and PCBs (Zakharov et al., 1997), in brown trout (*Salmo trutta*) living in polluted fluvial ecosystems (Sánchez-Gálan et al., 1998) and in damselflies (*Xanthocnemis xealandica*) exposed to fungicides and insecticides (Hardersen, 2000). While many studies have linked FA to stress in field studies, few studies have attempted to quantify changes in FA with changing levels of stress (e.g., to produce a dose-response curve), and those that have, have generated contradictory results (e.g., Valentine and Soulé, 1973, Mpho et al., 2001, Leamy et al. 1999, Polak et al. 2002).

By using chicks from the currently mandated avian reproductive test we tested whether there was a relationship between FA and dose or duration of exposure of their parents to one of four different herbicides, and if FA appeared to be a more sensitive marker of stress compared to standard reproductive endpoints measured from this test. Our results, based on measuring the lengths of the right and left ulna, tarsus and central toe from almost 1600 individuals, and then combining these values to create a composite index of FA

(FA^C), did not support these hypotheses. Chicks exposed *in ovo* to one of four different herbicides at three different concentrations, did not exhibit levels of FA that were consistently or predictably different from control birds, nor did dosed birds show any change in their developmental stability in relation to duration of pesticide exposure. Moreover, changes in FA^C were not observed in chicks even when they were exposed to an herbicide that caused significant reproductive effects. Unexpectedly, we did find that FA^C in control chicks from one of the four herbicides tested (herbicide one) did significantly increase in relation to duration of exposure. Variation in FA^C from control birds increased almost two-fold from the eighth to ninth week of sham exposure.

These results beg the question: why did our study fail to detect a positive relationship between FA and toxicant stress when several field studies have? One explanation is that internal egg concentrations of the herbicides might not have increased proportionately to the dose level given to the parents. Unfortunately, the current avian reproduction design does not mandate that egg residue levels be measured. It is also possible that the concentration of herbicide one through three that chicks were exposed to were not great enough to induce physiological stress capable of altering developmental stability. In the avian reproductive test, dosing levels are set to represent expected exposure in the wild, rather than trying to test the chemicals right up to initial maternal toxicity (as is the norm in mammalian reproductive tests), and herbicides generally tend to be of lower avian toxicity than insecticides or even fungicides (Mineau et al., 1994). Results reported by Valentine and Soulé (1973) appear to support this hypothesis. Valentine and Soulé (1973) reported a clear dose-response relationship in FA in pectoral fin rays of grunion fry exposed to increasing concentrations of p-p'-DDT (0.001 to 10 ppb). However, fry

exposed to similar doses appeared to have similar levels of FA. Control fry and those exposed to 0.001 and 0.01ppb p-p'-DDT had the lowest FA but they were all very similar (variance of right minus left values: 0.55, 0.52, 0.58). Fry exposed to 0.1, 0.4 and 0.7 ppb had greater FA than the first grouping, but again, their values were all very similar (variance: 1.5, 0.90, 0.92). Fry exposed to 1.0 and 10.0 ppb p-p'-DDT exhibited the greatest FA, but were also quite similar to one another (variance: 2.4, 2.9). If Valentine and Soulé's experiment had been designed to measure FA in fry exposed to concentrations of p-p'-DDT from only one of these three groups (e.g., control, 0.001, 0.01 ppb), then no notable dose-response relationship would have been observed.

This explanation for a lack of FA may be valid for birds exposed to herbicides one through three, but it is unlikely for the birds exposed to herbicide four considering that exposure to herbicide four caused marked reproductive effects. Since FA is hypothesized to increase with stress, it is surprising that chicks exposed to the fourth herbicide did not exhibit elevated FA over controls. It is possible that the lack of FA response in relation to the fourth herbicide could be related to reduced embryonic chick survivorship and chick survival to 14 days of age. It has been hypothesized that if developmental stability is related to individual quality, then the most developmentally unstable individuals are the ones most likely to be removed from the population during stressful conditions (Polak and Trivers, 1994). There is considerable evidence for such "developmental selection" to occur considering that FA often decreases in a population as mortality increases. Allenbach et al. (1999) showed that mortality in fishes exposed to the pesticides lindane and parathion (given at the LC_{70} dose) was related to individual FA. Western mosquito fish (*Gambusia affinis*) and sand shiners (*Notropis ludibundus*) were used in a time-to-death in 96-hour

toxicity test. Every three hours dead fish were removed from the aquaria and at the end of the 96 hours, the remaining fish were euthanized and FA measured in all animals. Results of the study indicated that the fish with the greatest average FA died earlier than fish with smaller average FA (Allenbach et al., 1999). Recently Polak et al. (2002) reported a reduction in FA in *Drosophila melanogaster* that was concomitant with high dose arsenite-driven mortality. For our study, only chicks euthanized at 14 days of age were measured for FA. If chicks that died *in ovo* or before 14 days of age had the greatest FA, then following the developmental selection hypothesis, chicks surviving to 14 days of age might have had the lowest FA in that population. Thus, no dose-FA-response relationship would be observed because FA variation in the cohort measured would have been very small. Even though our results appear to follow the developmental selection hypothesis, they cannot be used to support it. This explanation for a lack of FA response in quail chicks is only speculative. FA was not measured in dead chicks, so no direct relationship between mortality and FA can be established.

These explanations may account for the absence of FA in dosed birds, but do not explain the increase in FA in control birds from the first herbicide tested. Such seemingly random changes in FA may denote rearing conditions (e.g., eggs were artificially incubated) or other unknown factors. This is yet another concern in trying to use FA as a biomarker of chemical stress under "controlled" laboratory settings.

Our results did not support the hypotheses that FA would follow a predicable dose or duration of exposure response with pesticide exposure, nor did FA appear to be a more sensitive marker of stress compared to currently mandated reproductive endpoints measured in the avian reproductive test. Indeed, even marked reproductive deficits were not

accompanied or foreshadowed by higher levels of FA. Similar results generated from laboratory studies have also shown FA as an inconsistent biomarker of chemical stress (e.g., Premchand et al., 1998, Leamy et al., 1999, Polak et al., 2002). This irregularity appears to be a critical limitation to the use of FA as a reliable general biomarker of chemical exposure, in the lab and perhaps even in field based studies like the one conducted in this thesis.

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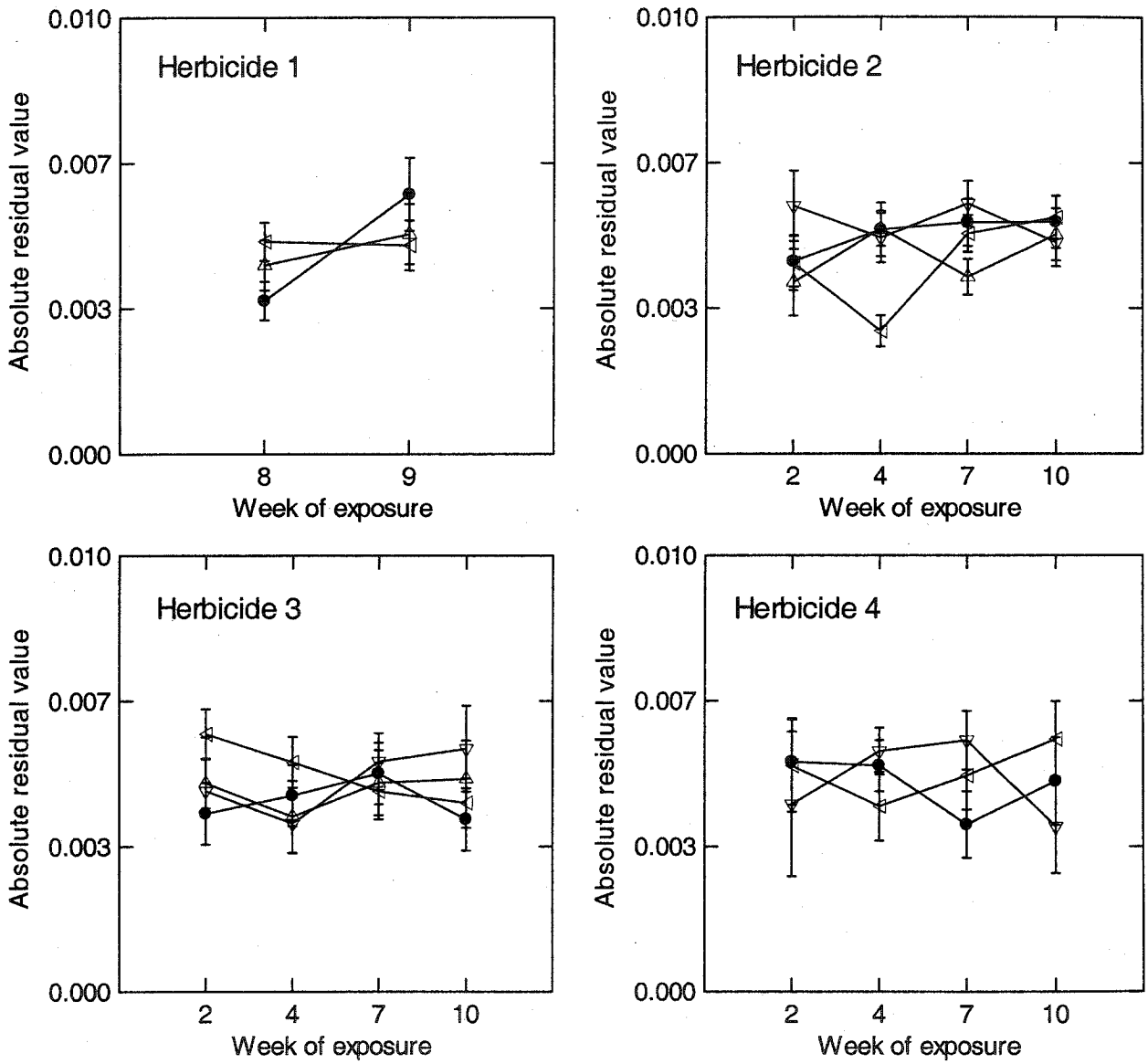
Figure Legends

Figure 1.: Absolute residual values (e.g., variance in FA^C used in Levene's test) from chicks exposed *in ovo* to all four herbicides, in relation to duration of exposure (weeks). Control chicks (•), chicks from low dose (∇), chicks from medium dose (◁), chicks from high dose (Δ). Values presented as means and SE.

Figure 1.


Herbicide 1
Herbicide 2
Herbicide 3
Herbicide 4
Absolute residual value
0.010
0.007
0.003
0.000
8
9
2
4
7
10
Week of exposure