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THE EFFECTS OF PESTICIDE USE IN APPLE ORCHARDS ON HEALTH AND REPRODUCTION OF CAVITY-NESTING BIRDS

By

CHRISTINE ANNETTE BISHOP, M.Sc.

A Thesis

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EFFECTS OF PESTICIDES ON BIRDS NESTING IN APPLE ORCHARDS

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ABSTRACT

In southern Ontario, Canada during 1988-1997, pesticide exposure and its effects on the immune and endocrine systems and the behaviour and growth of tree swallows (*Tachycineta bicolor*) and on reproductive success in tree swallows and eastern bluebirds (*Sailia sailis*) were studied. Birds were exposed in sprayed apple orchards and non-sprayed sites. There were significant effects of pesticides on all of these endpoints.

Sprayed tree swallow nestlings had significantly increased blastogenic response to pokeweed mitogen and delayed thymic maturation. Also, some tree swallow immune parameters were correlated to the date chicks were sampled. As the number of mixed sprays applied increased, there was a significant and positive increase in the concentration of the thyroid hormone, tri-iodo-thyronine, in male chicks and some indications of an increasing occurrence of a disrupted sertoli cell population on the seminiferous tubular basement membrane in testes.

There were no effects of pesticides on adult swallow incubation times. There were significant increases in hunger signalling by tree swallow chicks after organophosphorus insecticide (OP) spray events in 1996 and 1997 and, after a second OP spray in 1996, there were significant decreases in the number of feeding trips by parent birds. However, weight of chicks did not vary among sites due to pesticide exposure.

There was a significant increase in unhatched eggs in eastern bluebirds as organochlorine pesticide residues increased in eggs. At the gradient of contamination in tree swallow eggs, there were no trends between reproduction and organochlorines. With increasing toxicity and exposures to pesticides sprayed during 1988-1994, there were significant declines in egg fertility and daily survival rates of eggs and chicks of tree swallows and eastern bluebirds. Decreased

reproductive rates were more often detected in tree swallows than in eastern bluebirds however the possibility that organochlorine chemicals contributed to these effects in bluebirds cannot be discounted.

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People and institutions who have contributed significantly to the technical aspects of the thesis work are thanked within the acknowledgements of each chapter. However, for their important contributions and cooperation in all aspects of this thesis work I would like to thank the institutions and staff of the National Wildlife Research Centre, and the Laboratory Services Division, Pesticide and Contaminants Laboratory, University of Guelph.

I thank my supervisor James Quinn for always being supportive and providing insightful comments on the study design and results of the thesis work. My supervisory committee of Herman Boermans, Pierre Mineau, Ken Rosenthal and D.V. (Chip) Weseloh were also encouraging to work with. They were supportive yet constructively critical and were sincerely interested in the conservation value of this work. Pierre, in particular, was always helpful, enthusiastic and provided invaluable intellectual input to Chapters three and four. Herman Boermans and his technicians Anne Maslen, Dana Bruce and Navjoh Kaur provided critical laboratory support and creativity in the immunological aspects of the thesis.

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this study were crucial. Within his laboratory Ken Oakes, Sue Biddiscombe and Lisa Williams provided excellent quality laboratory support. Alice Hontela provided the thyroid hormone analyses and important critical comments on that part of the endocrine study.

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patience knows no bounds and she is always smiling. I deeply thank her for her enthusiasm, her brilliance, and her sincerity. It has been a joy to work with her and to get to know her lovely family.

Two friends that were always there to support me and to support this study because they believed in the conservation importance of the work were John Elliott and John Struger. John Elliott could empathise with my complicated lifestyle and difficulties of doing a PhD while conducting other work within a job at the Canadian Wildlife Service. Talking with John was always challenging and entertaining and always ended up taking my mind off the stress of the thesis. John Struger was always there when I needed to talk and his inner strength and calm were a perfect foil for me. Thanks so much to the two cool Johns!

I thank my parents, Marie (Boles) Bishop and Murray Winston Bishop, provided a very loving and stable family and, along with my sisters Andrea and Pamela and my brother Neil, they were always supportive of my endeavours. My parents never pushed their children into being anything but once you decided what you wanted they were there for you 100%. Since I was a little kid I was interested in issues like pollution and the environment. I know this was a source of amusement and interest to my father and his brother Art. Nevertheless, when it wasn't fashionable my parents recycled, re-used and composted. They took me to Silver Lake and uncle Frank's cottage and to uncle Murray's Pickwauket where I experienced nature in unique places. My mother was a nurse and dedicated to helping people. My father was a member of the Royal Canadian Air Force and a Prisoner of War. Now I recognize that my interest in helping 'the environment' and sticking with it when things were tough came from the strength and compassion of my parents. And I guess it was only natural with my interest in the environment and with the genes of my Potter and Bishop forebears, who lived in the Annapolis Valley where the apple orchards and industry were a big part of their life, that I would eventually come to study

the effects of pesticides on wildlife living in apple orchards.

DEDICATION

for Marie and Murray Bishop
and in the memory of Frank Boles

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LIST OF ABBREVIATIONS

ADI	Allowable Daily Intake
BSA	Bovine Serum Albumin
CARB	Carbamate
ChE or Che	Cholinesterase
DCFD	dichlorodihydrofluorescein diacetate
DDT	dichloro diphenyl trichloroethane
EBDC	ethylene bis-dithiocarbamate
EBI	ergosterol biosynthesis inhibitor
EROD	ethoxy resorufin-o-deethylase
FL	fluorescence
HBSS	Hanks Balanced Salt Solution
HCB	hexachlorobenzene
HCH	hexachlorocyclohexane
LD50	lethal dose to kill 50% of test organisms
NAA	napthaleneacetic acid
OP	organophosphorus insecticide
PBS-G	phosphate buffered saline-glucose
PCB	polychlorinated biphenyl
PCR	polymerase chain reaction
PHA	phytohemaglutinin-M
PWM	pokeweed mitogen
RPMI	Roswell Park Memorial Institute
T3	tri-iodo-thyronine
T4	tetra-iodo-thyronine

PREFACE

The concept for these studies was my own and based on my knowledge that many compounds were sprayed in orchards but there had not been in-depth examinations of the sublethal health and long-term reproductive effects of these compounds on birds in a natural situation. Therefore, I chose to include reproductive data for the period 1988-1993 in my thesis since I was involved in the collection of much of these data within my job at the Canadian Wildlife Service and these data had not been published previously.

For Chapters One, Two and Three, I conceived, designed, coordinated and conducted, along with field assistants, the field sampling portions of the study including liason with the farmers, and performed all statistical analyses and manuscript preparations. I assisted with the laboratory assays for sex hormones and for immunological assays described in Chapters One and Two. For Chapter Four, in cooperation with Pierre Mineau and Brian Collins, I conceived the approach to the statistical analyses. Brian Collins conceived the formulas to define egg fates and wrote and developed and ran the fortran and Statistical Analysis Systems programs for the analysis of the data. Neil Burgess, Mary Gartshore and William Read conducted the field sampling in 1988-1990. Under my supervision, William Read performed the field sampling in 1991-1994. With William Read, I conducted the field sampling in 1994. I performed the manuscript preparation for the reproductive data.

The thesis is written as a compilation of four separate papers although they are referred to as four chapters in the Table of Contents. Although each paper is presented in the style of the journal where it is submitted or will be submitted, these chapters share a common theme of the health and reproduction of songbirds nesting in sprayed apple orchards. There are a number of authors and contributors to each paper who have added significantly to the work. Their contributions are stated either within the acknowledgements or within the methodologies of each paper.

INTRODUCTION

Southern Ontario possesses an unusually temperate climate that provides a unique habitat for wildlife and optimum growing conditions for agricultural crops in Canada. These characteristics promote interactions between a wide diversity of wildlife and agricultural activities in this zone. The area is that part of Ontario located along the Lake Ontario shoreline or south of approximately 43° N latitude (Cadman et al. 1987). It is the transition zone between two major forest regions: the Eastern Deciduous Forest, which in Canada is commonly referred to as the Carolinian Zone; and the Great Lakes-St. Lawrence Forest (Cadman et al. 1987). Southern Ontario has warmer than average Canadian temperatures throughout the growing season. There is an ameliorating effect of Lakes Ontario and Erie on the climate which contributes to the existence of a frost-free period of 169 days, and mean annual precipitation of 700 mm which are ideal for tender fruit production (McCuaig and Manning 1982). Consequently, the floral and faunal assemblage in southern Ontario is diverse and includes many species that are at or near the northern or southern limits of their geographic range (Heagy and McHattie, 1995). Coincidentally these conditions present a valued land resource for agriculture, especially fruit production, due to the mild climate, and soils that are mainly class 1 and 2, well-drained and lightly textured (McCuaig and Manning 1982). Some bird species have thrived in the open landscapes typical of agricultural areas while other species persist despite the removal 90% of the forest cover of southern Ontario for agricultural and urban development (Cadman et al. 1987; Kirk et al. 1996). Consequently, wildlife that occur in Ontario are forced to live in wild remnant and fragmented habitats on or near farms and cities or in semi-natural areas such as orchards. Since pesticide use is common in apple orchards in southern Ontario, the purpose of this thesis was to study the impacts of these chemicals on the reproduction and health of songbirds that nest in orchards in southern Ontario.

By 1791, 200 000 inhabitants were established in agricultural settlements along the Niagara Peninsula in southern Ontario (McCuaig and Manning, 1982). By the later 1800s, pesticide use on larger orchards was common. 'Bordeaux' mixture consisting of 0.3 lbs/10 gallons water each of copper sulfate, and lime was used as a fungicide for apple scab (*Venturia inaequalis*) (Beckett, 1913; Emerson et al., 1945). Two arsenic sprays, Paris green(0.33 lbs/ 50 gallons water) and lead arsenate (1.5 lbs/50 gallons water), were used on orchards to control insects, mainly codling moth (*Cydia pomonella*) (Emerson et al., 1945). Spray applications were made from two to seven times per growing season to control the same pests of concern to apple growers today (Ontario Ministry of Agriculture and Food, 1994). By the 1940s, agricultural publications began describing methods by which these chemicals could be applied efficiently to improve yield and reduce spray costs (Woodworth and Rawlings, 1945). Lead arsenate was still being used but lime-sulfur (0.33 gallon/ 10 gallons water) was beginning to replace Bordeaux mixture because some apple varieties were tarnished by copper sulfate (Emerson et al., 1945).

Around the turn of the century, the use of orchards by birds and the economic value of birds to agriculture and, in particular, to orchards were also being studied. Beginning in the 1880s, ornithologists in North America were noting the regular use of orchards by birds and the value of birds, especially woodpeckers, in the control of codling moth. Downy (*Picoides pubescens*) and Hairy (*Picoides villosus*) woodpeckers consumed the overwintering codling moth larvae (MacLellan 1958; Kirk et al., 1996). Some birds were also considered pests in orchards and studies on this issue were also common (Kirk et al., 1996). The study of interactions between orchards and bird communities was prominent at the turn of the century but declined in the 1930s and 1940s. This was partly due to the increasing use of pesticides especially the introduction of DDT and its related compounds in the late 1940s which appeared to provide efficient and ideal pest control (Kirk et al 1996). The extensive use of organochlorine compounds continued in orchards through to the 1970s, and the value of birds

to agriculture was all but forgotten (Kirk et al. 1996). During this period, the intensity of DDT (50-60 lbs/ acre/year) use in orchards on the Niagara Peninsula was extremely high (Ginsberg and Reed, 1954).

By the 1970s, the scientific focus on birds in agricultural areas became the study of the effects of pesticides on birds. Organochlorine pesticides were causing death of adult birds and other wildlife as well as embryotoxicity and eggshelling thinning in birds (Newton et al. 1986). Most organochlorine pesticides (OCs) were banned from use in North America by 1972, except endosulfan, which remains the only organochlorine compound in use today in orchards in Ontario. In the latter 1970s and 1980s, chemicals that are less persistent than the organochlorine compounds were introduced to control the major pests of apple development. Some of the new compounds such as the organophosphorus and carbamate insecticides are much more acutely toxic than the OCs whereas others such as the synthetic pyrethroids have low acute toxicity to vertebrates but high acute toxicity to insects. Fungicides and acaricides have been developed which have low persistence, and low acute toxicity to vertebrates but are effective in pest control. Despite the introduction of these new chemicals, to this day, bordeaux mixtures are occasionally used and sulfur is commonly used in some orchards to control fungi of apples.

Apple orchards now occupy about 12565 ha of land in Ontario (Statistical Services Unit, 1992). Although there are two commercial organic orchards in Ontario, all other orchards use pesticides to control apple pests. Chemicals are applied on a weekly basis during early April to mid-August in Ontario. Applications are often made as mixtures of insecticides and fungicides. Among the sites I studied, I observed 30 species of birds nesting or feeding in orchards and eight species of amphibians breeding in ponds within orchards during 1994-1997. This investigation was conducted because there are few long term studies of avian reproduction in wild birds exposed to pesticides and little understanding of the impact of pesticides on health and behaviour of wild birds and the impacts

on their food resources. To test for effects of pesticides on songbirds, I chose the tree swallow (*Tachycineta bicolor*) and the eastern bluebird (*Sialia sialis*) as model species because they occasionally nest in natural cavities within orchards and they readily occupy nest boxes placed in orchards and non-sprayed sites. The use of nest boxes standardized exposures to pesticides which facilitated the study of the health and reproduction of these birds in relation to pesticide use in the apple orchards.

The results of this study have implications for avian survival and conservation efforts in Ontario. The tree swallow is representative of an aerial insectivore which maintains a high metabolic rate relative to ground feeding birds (Williams, 1988). The eastern bluebird is representative of ground foraging birds and produces more than one brood per year whereas tree swallows in Ontario produce only a single brood (Robertson et al. 1992; Peck and James 1987a;b). Nest boxes are often placed in rural areas to attract eastern bluebirds due to concern for the decline in their populations in the past few decades (Read and Alvo, 1995). Moreover, species such as tree swallows, house wrens (*Trogolodytes aedon*) and black-capped chickadees (*Parus atricapillus*) commonly use these boxes and, less commonly, other species such as great crested flycatcher (*Myiarchus crinitus*), purple martin (*Progne subis*) and house finch (*Carpodacus mexicanus*) also use nest boxes meant for eastern bluebirds (McNicholl et al. 1994).Therefore, songbirds are not only potentially exposed to pesticides when they nest naturally in agricultural areas but may also be unintentionally exposed by well-meaning naturalists who often place bluebird box trails in rural areas where pesticides are used (McNicholl et al. 1994).

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Chapter One.

Health of Tree Swallows *(Tachycineta bicolor)* Nesting in Pesticide-Sprayed Apple Orchards in Ontario, Canada

Immunological Parameters

Health of Tree Swallows *(Tachycineta bicolor)* Nesting in Pesticide-Sprayed Apple Orchards in Ontario, Canada

I. Immunological Parameters

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ABSTRACT

The degree of pesticide exposure and its effects on the immune system and its development were determined in 16-day old tree swallow (*Tachycineta bicolor*) chicks from four sprayed apple orchards and three non-sprayed sites in southern Ontario, Canada during 1994-1995. Persistent contaminant residues were measured in tree swallow eggs and in each chick hepatic ethoxyresorufin-o-deethylase (EROD) activity; body, immune organ and liver masses, lymphocyte blastogenesis response, respiratory burst and phagocytic responses, hematological evaluation, histological development of thymus, bursa of fabricius, and spleen were determined.

Chemicals sprayed on apple orchards were mainly ethylene-bis-dithiocarbamate and myclobutanil fungicides and organophosphorus, carbamate and synthetic pyrethroid insecticides. During the period between oviposition of the first egg in each nest to the sixteenth day after hatching, individual nests in orchards were exposed to between four and eleven individual chemical applications and up to three mixtures of pesticide sprays. Concentrations of pesticides, PCBs, lead and arsenic residues in tree swallow eggs and liver were low and not variable among sites except pp'DDE which was as high as 2.29 ug/g wet wt. in eggs. EROD activity was not different among sites. Organochlorine and trace metal residues and EROD activity were not correlated with any immune parameter.

In sprayed birds, we found a significantly increased blastogenic response to pokeweed mitogen (12.5 ug/ml). However, nests were initiated over a period of several weeks and we also found changes in other tree swallow immune parameters which were related to the date of chick collection. Hematological parameters, bursal and thymic masses, phagocytic response and thymic development were all correlated with the day the chicks were 16 days of age. After accounting for the collection date of birds from each nest, we found cell proliferation in the cortex and delayed thymic involution correlated positively with increasing spray exposure. We also found that birds in sprayed orchards were slightly anemic compared to birds from non-sprayed sites and there was smaller bursal masses and an increase in relative heterophil concentrations in the sprayed orchard birds. The local inflammation may have been caused by trematode parasite infections although pesticide exposure also correlated positively with these parameters.

This is the first study of the immunology and effects of current pesticide exposures in wild passerines therefore it is difficult to predict the long-term consequences of the apparent stimulated immune systems in sprayed birds. However, some environmental contaminants that overtly stimulate the immune system in mammals have induced hypersensitivity and/or autoimmunity. Therefore we speculate that our these effects are possible in tree swallows.

INTRODUCTION

Birds which nest or feed in apple orchards are frequently exposed to a wide variety of pesticides (Blus et al., 1987; Patnode and White, 1991; Fleutsch and Sparling, 1994). For example, organophosphorus (OP) and/or carbamate insecticides are applied up to four times and fungicides are applied bi-weekly during an average apple growing season in Ontario, Canada, where this study was conducted (OMAFR, 1994). These pesticides are often applied as mixtures of chemicals. There is limited understanding of the effects of pesticide mixtures although additive toxicity is common and some chemical combinations are synergistic in their toxic effects (Thompson 1996). The dithiocarbamate and ergosterol biosynthesis inhibiting fungicides used in orchards can alter liver enzyme activity (Rivière et al., 1984; Plant Industry Directorate, 1993; Johnston et al., 1994) which can potentially increase the toxicity of other pesticides when exposure to mixtures of pesticides occurs (Johnston et al., 1994; Thompson, 1996). In tree swallows, Bishop et al (1998) found liver enzyme induction as measured by EROD activity was sensitive to both polychlorinated biphenyls (PCBs) and pp'DDE. Others have found EROD activity to be sensitive to pesticide exposure in rats (Bunyan et al., 1972; Krechniak et al., 1994). In addition, bird eggshells are permeable to organophosphorus and dithiocarbamate compounds and some are teratogenic through this route of exposure (Hoffman, 1990). Past use of organochlorine pesticides, especially DDT, and lead arsenate pesticides in orchard still exposes wildlife to these persistent and bioaccumulative contaminants (Johnson et al., 1976; Elliott et al., 1994).

Exposure to the types of pesticides used in apple orchards can impact on reproduction and thermoregulation of birds (Rattner et al., 1982; Fleutsch and Sparling 1994). But there has not been any examination of the immune and/or endocrine systems of songbirds exposed to pesticides in the wild although environmental contamination has been implicated in endocrine dysfunction in wildife (Colborn and Clement, 1992). Lab studies have shown that OPs, carbamate

and organochlorine insecticides, dithiocarbamate fungicides, and lead can induce alterations in immune responses and tissue development in acutely and chronically dosed rodents and poultry (Dean and Murray, 1991; Pruett et al., 1992; Pruett 1994). Immunosuppression has been demonstrated in wild birds exposed to pollutants such as selenium (Fairbrother and Fowles, 1990) and polychlorinated biphenyls and organochlorine pesticides (Grasman et al., 1996). Alterations in parts of the immune system have also been documented in humans who are occupationally exposed to pesticides in agricultural areas (Repetto and Baliga, 1996). Therefore, the frequency and degree of chemical exposure and the immune systems of tree swallow chicks *(Tachycineta bicolor)* from nests within sprayed apple orchards and non-sprayed sites were studied. Exposure was measured by analysis of tissues for pesticides and trace metals, liver enzyme activity(EROD), and calculating the number of times each orchard nest was exposed to individual and mixtures of pesticides sprayed during egg and chick development.

METHODS

Study Areas

In total, four sprayed apple orchards and three non-sprayed sites were studied during 1994 and 1995 but only three sprayed orchards were studied in each year. All sites were located in the Great Lakes basin in southern Ontario, Canada (~ latitude 43°15'N/ longitude 80°2'W). There are only two non-sprayed, mowed orchards in Ontario therefore using these sites would not have been practical for the study. One non-sprayed site and two sprayed orchards were located within the Hamilton Harbour watershed while the rest were located in the Grand River watershed. Among the control sites, one was a non-sprayed, mowed orchard located on the lands of the Hamilton Region Conservation Authority. To our knowledge, this site had never been sprayed with organochlorine compounds and had not been sprayed with any pesticide since 1987. The other two non-sprayed sites were cattle pastures where pesticides had not been sprayed for at least ten years. The cattle maintained the vegetation at a low height (~0.1-0.25 m) which was

similar to the height of the herbaceous vegetation within an orchard. The sprayed apple orchards were mowed mechanically between tree rows and at three of four sites, herbicide was applied once a month to vegetation between the trees. All sites had ponds (0.5-1 ha) or watercourses within 500m of the nest-box area.

Tree swallows will nest in natural cavities in orchards (Bishop, unpublished), however, to standardize exposure and nesting conditions among sites, 25-40 pine nest boxes, unpainted on the inside of the box, were installed in each study site and this species readily occupied these artificial nest sites. Each nest box was 8- 10m from its nearest neighbour and entrances to all nest boxes faced south to avoid the prevailing west wind. Since tree swallows never perch on poles for nest boxes, nest predators were deterred by applying axle grease to nest poles a week prior to the start of breeding season. The nest boxes in the orchards were located between apple trees. All sites had nest boxes installed at least six months prior to the the breeding season. During the egg laying and hatching periods, nests were checked every day whereas during incubation and chick-rearing, nests were checked every two to three days. Therefore, hatch date used to determine the age of the chicks was accurate to within 24 hours.

Field Sampling Design

From four to ten nests per study site, two fresh eggs per nest were collected during the period 1994-1997 (Table 1). Egg contents were analyzed for a suite of organochlorine pesticides and PCBs. Sixteen-day old chicks were sampled for immunological assessment from 3 to 9 nests per site. In total, the sample size from sprayed orchards was 23 nests in 1994 and 22 nests in 1995. From non-sprayed sites, the sample size was 9 nests in 1994 and up to 15 nests in 1995 (N= 14 for histopathology whereas N=15 for immune assays and body and organ masses).

Histopathological assessment was conducted on one chick from each nest sampled in 1994 and 1995. From the same nests sampled in 1995 for histopathology, two sibling chicks were collected for immune assays (blastogenesis; respiratory burst; phagocytosis), body and organ

masses and EROD activity. Body mass is a good predictor of first year survival of fledging chicks (McCarty, 1995) therefore it was chosen as a general indicator of adequate development of chicks. Of those chicks collected for immune assays, whole blood was collected from one chick per nest (not all nests were sampled) for hematological measurements. Chicks were collected at approximately 0730 hrs which was prior to spray events on that day. Therefore, calculated exposures were based on spray events occurring until the end of day 15 of development in each brood.

Weather Conditions

Minimum and maximum air temperatures during the sampling periods were 4.8°C and 34.5° C during 12 June to 2 July 1994 and were 6.1°C and 34.5°C in 9 June to 23 July 1995 (Environment Canada, 1994; 1995).

Tissue Collection and Sex Determination

Birds were transported from the field sites to the laboratory at University of Guelph, Ontario (25 km; 30 minute travel time) where blood sampling and all immune assays and hematological assessments were performed. Blood was collected from birds by jugular venipuncture approximately 20 minutes after birds were delivered to the laboratory. Blood (1-1.2 ml per bird) was collected using a 27-1/2 gauge syringe and sterile techniques. Immediately after blood collection, birds were killed using carbon dioxide asphyxiation. Within 20 seconds of blood collection from the bird, approximately 500 ul whole blood for hematology was mixed with heparin. Blood was stored on ice until hematological analyses took place within 2 hours of blood collection. The remainder of the blood (about 1 ml) was split equally and used for phagocytosis and respiratory burst assays which were initiated within 10 minutes of blood collection.

Within ten minutes after asphyxiation of the birds, liver and spleen were removed. Liver was immediately weighed and then placed in liquid nitrogen (-80 °C) for EROD activity analyses. Spleens were either stored in 10% buffered formalin for histopathology or prepared for

blastogenesis assays. Thymus and bursa were removed within 3 hours, weighed and then stored in formalin for histopathology. Mass of whole body, liver, spleen, bursa and thymus were measured to the nearest 0.00001g with a Mettler AE 100 analytical balance. All blood and tissue samples were coded with generic identification numbers to facilitate 'blind' laboratory analyses.

Sex of each chick was determined by visual inspection for testes. If the testes were not present, the chick was designated as a female. To validate that method, blood samples from putative male and female chicks (N=15 in total) and from male and female adult tree swallows (N= 3 ♀; N=2♂; adult blood sampled for a related study, see Bishop et al., this volume) were analyzed by polymerase chain reaction (PCR). To perform sexing by PCR, 1 ul blood was mixed with ethanol and subjected to protease deoxyribonucleic acid (DNA) isolation for 30 min. A PCR cocktail was added and amplification of DNA was done. The PCR products were run on an agarose gel and each chick sample was run concurrently with adult male and female swallow blood and a negative control sample. PCR analysis was performed by PE Zoogen Inc. It was found that sexing by inspection for presence or absence of testes agreed with the PCR results in every case (data not shown). All birds were handled according to the Canadian Council on Animal Care (Olfert et al., 1993) and procedures were approved by the Canadian Wildlife Service Animal Care Committee.

Chemical Exposure

Chemical Applications in 1994 and 1995

In all sprayed apple orchards, multiple types of pesticides and spray events occurred during the approximately 35 day period from the first egg laid per nest through egg incubation to the day before hatching (defined as the 'egg stage'), to hatching and development to 16-day old chicks (chick-rearing stage) (Peck and James, 1987). Each farmer provided the exact date of spray, name of the chemical and its application rate for each orchard (Table 1) which was consistent with the recommendations published by the provincial government (OMAFR, 1994).

Pesticides in were applied with an air-blast sprayer by the orchard owners who are licenced pesticide applicators. The chemicals were applied according to the farmer's choice of spray schedule which depended on pest cycles in the orchard and advisories from the Ontario Ministry of Agriculture and Rural Affairs (OMAFR) 'agri-phone' and Fruit Production Recommendations (OMAFR, 1994). Therefore these study sites were generally representative of chemical application methods and schedules used in apple orchards in Ontario.

Because pesticides can diffuse through avian eggshells (Hoffman, 1990) exposure to the embryo could occur as soon as eggs are laid. Therefore, the number of times each nest was exposed to a chemical application during each of the egg and chick-rearing stages was determined. For those periods, the number of occasions each nest was exposed to two or more chemicals applied as a tank mixture or to two or more chemicals sprayed sequentially on the same day (mixtures or mixed spray events) was summed.

EROD Activity

Liver homogenates were prepared according to Pyykkö (1983). The left lobe of the liver was chopped then homogenized in four volumes of buffer (0.1 M phosphate, pH 7.4) using six passes at 750 rpm (Heidolph RZP-2000 homogenizer). The tissue was kept on ice during the entire procedure. Cell debris was removed by centrifuging for 17 min at 12,000 g. Microsomes were prepared by gel filtration, aliquotted into small vials and immediately frozen in liquid nitrogen. Samples were subsequently thawed on ice and mixed gently. Treatment plates contained 50 µL of 10mg/mL bovine serum albumin (BSA), 25 µL of microsomes (about 100 µg of protein per well) and 20 µL of 50 µM ethoxyresorufin dissolved in (1:9) MeOH : 0.1 M TRIS-NaCl (pH 8). The samples were preincubated (37°C) for 5 minutes on an agitator before 25 µL of freshly prepared and chilled 25 µL NADPH generating system (64 mg NADP, 258 mg isocitrate, 80 mg nicotinamide, 60 mg $MgCl_2$ 400 µL isocitrate dehydrogenase and 4.6 mL 0.1M TRIS-NaCl (pH 8) was added to each plate to initiate the reaction. After an incubation period of 10 minutes,

1 mL of chilled methanol was added to each plate to terminate the reaction. Fluorescence levels were measured using the Cytofluor 2300 Fluorescence Measurement System with an excitation wavelength at 530 nm and an emission wavelength at 590 nm. Blanks contained no NADPH generating system. The amounts of resorufin formed in the reaction wells were calculated with a Rhodamine B standard curve, but the results were then converted to the amount of resofurin formed using a conversion factor (Burke et al., 1977). Microsomal protein concentrations were determined according to the procedure outlined by Peterson (1977). Enzyme activity is represented as a mean of three replicate plates and is expressed as pmoles per minute per milligram of microsomal protein. The lower limit of detection for EROD activity was 4 pmol/min/mg protein.

Contaminant Residue Analysis

Organochlorine pesticides and PCBs

Methods for measuring chlorobenzenes and total PCB congeners (PCBs) and organochlorine pesticides (OC) except endosulfan (see below) are based on those of Peakall et al. (1986) and Bishop et al. (1996). Briefly, 5 g of a pooled egg sample was ground with 25 g sodium sulphate, poured into a 2.1 cm internal diameter chromatography column and eluted slowly with 300 mL hexane. The extracts were reduced to approximately 5 mL in a rotary evaporator and fractionated by Florisil chromatography into 3 fractions using varying concentrations of hexane. Each fraction was reduced to less than 2 mL in a rotary evaporator and made up to a suitable volume for analysis by gas chromatography using a Hewlett Packard 5840 gas chromatograph equipped with a splitless injector port and a DB5 column (dimensions: 0.25 mm internal diameter x 60m length). Fraction 1 was analyzed for residues of octachlorostyrene, chlorobenzenes, mirex, photomirex and PCBs. Total PCBs were estimated by determining the sum concentration of all individual PCB congeners measured. These included PCB congeners: #28, 31, 42, 44, 49, 60, 64, 66/95, 70, 74, 87, 97, 99, 101, 105, 110, 118, 129,

137, 138, 141, 146, 149, 151, 153, 158, 170/190, 172, 174, 180, 182/187, 183, 185, 194, 195, 200, 201, 203, 206 (Ballschmiter and Zell, 1980). Fraction 2 was analyzed for all chlordanes, nonachlors, and cyclohexanes, DDT, DDD and DDE and fraction 3 was analyzed for dieldrin and heptachlor epoxide. Detection limits for wet weight OCs, chlorobenzene and PCB residues were 0.0001 ug/g.

Endosulfan

The samples were blended with acetonitrile: water and then partitioned with methylene chloride. A Florisil cleanup of extracts was performed prior to quantitation on Hewlett Packard 5890 Series II gas chromotograph equipped with an electron capture detector. Recoveries of standards were 85-100%. Detection limit for endosulfan was 0.002 ug/g.

Lead and Arsenic

As part of a related study (Bishop et al., this volume), 16-day old male tree swallows were collected and sacrificed in 1996 from the six study sites used in 1995 in this study. Livers from one male chick from each nest from two to six nests per study site were analyzed for lead and arsenic. Livers from each site were pooled and analysed as a single sample. Methods of analysis are described in Bishop et al (this volume).

Histopathology

Tissues fixed overnight in buffered formalin at room temperature were later embedded in paraffin wax. Serial sections (5 µm) were cut and stained with hematoxylin and eosin and examined under a 100 power light microscope. Only one cross-section from each organ was evaluated. Histological assessments were conducted by one observer.

Five aspects of thymus histopathology were assessed, scored and statistically analyzed in the following manner: 1) thickness of cortex (absent or thin vs. moderate to thick) 2) tingible body macrophages in cortex and medulla (none vs few to many) 3) cortical lymphocyte density (low vs. moderate to high) 4) presence or absence of heterophils 5) and an overall evaluation of

degree of involution of the thymic tissue (none vs. mild, moderate or marked involution).

For the spleen the following evaluations were made: 1) in the T-dependent white pulp the degree of development of the periarteriolar/periellipsoidal sheaths was made (absent vs. moderate to thick) 2) number of follicles in the B-dependent white pulp (none vs. few to many) 3) overall involution of spleen cell organization (normal vs. abnormal such as depleted and involuted and normal vs hyperplastic).

The bursa of fabricius was evaluated by examining: 1) in the epithelium, overall degree of inflammation (present vs absent); mucous metaplasia (present vs. absent) squamous metaplasia (present vs. absent) 2) in follicles, cellular density (low vs. moderate to high); apoptosis (low vs. moderate/high); follicular cysts (none vs. few to many) 3) presence or absence of parasites 4) involution of the bursa (normal vs. involution is mild, moderate or marked).

Blastogenesis

Using sterile techniques, spleens were removed from chicks and weighed. Then single cell suspensions of splenic cells were prepared by forcing the spleen through a fine mesh and into Hanks Balanced Salt Solution (HBSS). Spleen cells were washed twice with HBSS. The spleen cells were recovered by centrifugation at 250g for 10 minutes at 23°C. Cells were resuspended in supplemented medium RPMI-1640 (Roswell Park Memorial Institute). A cell count was performed in duplicate using a hemocytometer and cell concentrations were adjusted to 5×10^6 spleen cells per ml in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 u/ml penicillin, and 100 ug/ml streptomycin.

Proliferative assays were carried out in flat-bottomed culture plates as described for chicken lymphocytes (Hovi et al., 1978; Vanio and Ratcliffe, 1984). For tree swallows, triplicate cultures of lymphocytes were stimulated with optimal concentrations of the mitogens concanavalin A (30 ug/ml), pokeweed mitogen (PWM) (12.5 ug/ml), phytohemaglutinin-M (PHA) (12.5 ug/ml), and lipopolysaccharide (LPS)(50 ug/ml from *Escherichia coli* erotype O26: B6).

Cells were cultured at 37°C in a humidified atmosphere with 5% CO_2 in 95% air environment for 90 hours before harvesting and were pulsed with 0.5 µCi tritium-labelled (^{3}H) thymidine per well during the last 18 hours of culture. Triplicate unstimulated control cultures were included for each animal for each day of harvesting and control counts were subtracted from treatment groups before statistical analysis. Proliferation was quantified by measuring the incorporation of ^{3}H-labelled thymidine.

Respiratory Burst

The methodology followed was that of Ochs et al. (1988) with some modifications. Diluted blood was layered onto Histopaque-1077(Sigma-Aldrich Canada) and centrifuged 20 minutes at 100g (20°C). White blood cells were collected and washed twice with HBSS and centrifuged 200g for 8 minutes at 4°C and then resuspended in phosphate buffered saline and glucose (1g/l) RPMI (PBS-G). A cell count was performed in duplicate using a hemocytometer and cell concentrations were adjusted to 2 x 10^6 cells/ ml in PBS-G and kept on ice. Cells plus PBS-G (1 ml) were mixed with 1.25 ul of 4uM DCFD (dichlorodihydrofluorescein diacetate) mixed and incubated 15 minutes in a 41°C water bath while protected from light. PMA (phorbol myristate acetate) (100ug/ml) was added to a final concentration of 200 ng/ml, solution was vortexed and returned to water bath. Lymphocytes were removed by gating and fluorescence (FL) was measured using flow cytometry (Coulter XL). Optimal fluorescence was found after 70 min. The net fluorescence (FL stimulated - FL resting cells) was calculated where FL is measured at 70 min. Fluorescence of resting cells was determined at the 0 min. time point prior to PMA addition to the cell solution.

Phagocytosis

Blood was diluted and mixed at 1:1 with HBSS. The mixture was layered on Histopaque-1077 and centrifuged 100g for 20 minutes at 20°C until free of red blood cells. White blood cells were collected from the interface and washed twice with HBSS and centrifuged at 200g for 8 min

at 4°C. Cells were resuspended in RPMI 1640 and kept on ice. Cell concentration and viability was determined using a hemacytometer and concentration was adjusted to 2 x 10^6 cells/ml. Cells (1ml) were incubated in a 41°C water bath for 90 minutes. After 30 minutes, fluorescent latex beads were added at a ratio of 100 beads per cell, the suspension was mildly agitated and returned to the water bath. After incubation, 0.5 ml of cell suspension was layered over 4 ml centrifugation gradient (RPMI 1640 plus 3% BSA) and centrifuged at 150g for 8 minutes. The supernatant was aspirated and cell pellet was resuspended in 500 ul 0.5% paraformaldehyde in PBS and stored at 4°C. Immediately prior to analysis, cells were lightly vortexed. Lymphocytes were removed by gating and fluorescence was measured within two hours using flow cytometry (Coulter XL). Results are expressed as percentage of the fluorescence of phagocytic cells with three or more beads relative to the total fluorescence adjusted for fluorescence of red blood cells in each sample.

Hematology

At the clinical pathology laboratory of the Ontario Veterinary College, University of Guelph, the following parameters were determined in whole blood samples (25 ul): total white cell count and differential cell count (heterophils, lymphocytes, monocytes, basophils, and eosinophils), erythrocyte count, hemoglobin concentration, and hematocrit . Mean corpuscular volume of erthryocytes was also determined. Classifications of cell types followed Dein (1984). Erythrocytes were counted by performing a differential count of the number of nucleated red cells per 100 leukocytes on a Wright-stained whole blood smear. Hemoglobin was determined by diluting whole blood with Zap-a-globin ® lyzing solution (1:500), centrifuging at 1000g for ten minutes and reading the supernatant with a Hemaglobinometer®. Counts of heterophils, lymphocytes, monocytes and eosinophils were performed using the UNOPETTE Test 5856 *in vitro* diagnostic reagent system (Becton-Dickinson Co.) using 3% acetic acid as the diluent. Mean corpuscular volume was measured by lazer using a Technicon HI (Bayer Diagnostics).

Statistical Analysis

Data were log-transformed, where necessary, to meet normality and homogeneity of variance requirements for parametric analysis. If those criteria could not be met, non-parametric tests were used. All statistical analyses were considered significant at $p \leq 0.05$ (Sokal and Rohlf, 1981) except correlations between immunological parameters and collection dates and spray exposures where $p=0.01$ was used due to the large number of correlations performed. All correlation results found to be significant were examined by scatterplots to confirm credible gradients in results occurred. All data were analyzed using STATISTICA for Windows (Statsoft, 1997).

The number of individual spray events and number of mixtures of chemicals to which each nest was exposed was compared among sprayed orchards using the Kruskal-Wallis and Tukey's test for unequal sample sizes (Sokal and Rohlf, 1981). For the two orchards sampled in both years, spray exposures per nest were compared between 1994 and 1995 using the Mann-Whitney test (Sokal and Rohlf, 1981).

From each study site, only one pooled residue analysis was performed on each tissue therefore this precluded statistical comparisons among sites for organochlorine compounds, lead and arsenic. Differences in concentrations of residues between spray treatment groups (sprayed orchards vs. non-sprayed sites) were determined using the Mann-Whitney test (Sokal and Rohlf, 1981).

For histopathology, results were compared between birds from sprayed and non-sprayed sites using the Chi-square test with Yates' correction (Sokal and Rohlf, 1981).

Because nests were initiated at different dates, 16-day old chicks were not collected on the same dates within and among sites. Therefore, the spray exposures also varied among nests during egg and chick development. Product-moment or Spearman Rank correlations, or logistic regressions were performed to examine the trends between the number and type of spray

exposures applied in 1994 and 1995, and persistent contaminant residues in chicks versus immune parameters and body and organ masses (Sokal and Rohlf, 1981; Fox, 1984).

The importance of collection date of chicks and/or weather conditions correlations with immune parameters was also investigated. To do this, correlation or logistic regression analysis between chick collection date and maximum air temperature on the day prior to chick collection was performed. In cases where collection date as well as spray exposures correlated with an immune parameter and multicolinearity between spray exposure and collection date did not occur, multiple regression analysis was performed to determine the significant independent variables (Sokal and Rohlf, 1981).

Mann-Whitney tests were used to determine if there were differences in immune parameters, body and organ masses between sexes or sibling chicks within sprayed and non-sprayed groups. Nested Analysis of Variance (ANOVA) for unequal sample sizes or Kruskal-Wallis tests and Tukey's test for unequal sample sizes were used to compare parameters among spray treatments and sites (Sokal and Rohlf, 1981). To adjust for differences in collection dates among nests when necessary, parameters were compared among sprayed and non-sprayed sites using Analysis of Covariance (ANCOVA) (Sokal and Rohlf, 1981). Least-squared means tests were applied to determine differences in results among sites.

RESULTS

Weather Conditions

For 1994, air temperatures were significantly and negatively correlated with collection date (Spearman Rank R = -0.41; p=0.04 maximum air temperature on day 15). In 1995, maximum air temperature on the 15th day of age for chicks significantly increased with collection date (Spearman Rank R = 0.79; p=0.001 maximum air temperature on day 15). It was felt that collection date would best represent weather and any unmeasured factors specific to the collection date. Therefore, chick collection date was tested in the correlations with each immune

parameter and spray exposures.

Chemical Applications

There were 20 chemicals sprayed and 13 types of mixtures applied in the orchards during 1994 and 1995 (Table 2). In 1994, between oviposition of the first egg to the day before collection of chicks at 16 days of age, individual nests in sprayed orchards were exposed to a minimum of four and a maximum of 11 individual spray events and between one and three mixtures of chemicals. During 1995, the range in exposure per nest was four to 11 individual spray events and one to three mixtures of chemicals per nest (Table 3). There were six types of mixtures applied in 1995 that were not applied in 1994 whereas four mixtures were applied in 1994 and not applied in 1995 (Table 3).

There were some significant differences in the number of spray exposures in orchards in each year (Table 3). However, the number of individual and mixed spray events were fairly consistent between years. During the egg stage, approximately four individual chemical applications and one to three mixed sprays were applied (Table 3). During chick-rearing, approximately three individual applications and mixtures were not often sprayed but usually at least one mixed spray exposure occurred (Table 3).

In 1995, the number of individual chemical applications per nest during chick development was positively correlated with collection date whereas exposure to individual sprays and to mixtures per nest during egg development were negatively correlated with collection date (Table 4). This reflects the pattern of spraying more often and more mixtures in early May. In contrast, in 1994 there were no correlations between collection date and individual spray applications (Table 4).

EROD Activity

Mean EROD activity in livers ranged from 10.7 to 34.9 pmol/min/mg protein among sites. Differences among sites were not significant. There were no significant correlations among

EROD activity and spray applications, pp'DDE concentrations, any immune parameter, or total PCB (Table 4).

Contaminant Residues in tissues

With the exception of pp'DDE, concentrations of organochlorine pesticides were low and not variable among sites. Among sites, concentrations of 1245- and 1234-tetrachlorobenzene, pentachlorobenzene, α-, β- and γ- hexachlorocyclohexane, hexachlorobenzene, octachlorostyrene, heptachlor expoxide, oxy-, trans-, cis-chlordane, dieldrin, op'DDE, op'DDD, op'DDT, pp'DDD, pp'DDT, cis- and trans-Nonachlor, photomirex, mirex and endosulfan ranged from non-detectable (<0.0001ug/g or 0.002 ug/g) to 0.0568 ug/g wet wt. Concentrations of pp'DDE were 0.66 to 2.29 ug/g among sprayed orchards and 0.36 to 2.23 ug/g among non-sprayed sites (Table 1). Total PCB concentrations in eggs were 0.27 to 0.49 ug/g among all sites except Orchard Site 2 where eggs contained 0.61 ug/g. There were no significant differences in organochlorine pesticides and PCB concentrations between sprayed and non-sprayed treatment groups. Hepatic lead and arsenic levels were also extremely low and did not vary among sites (Bishop et al this volume). Since concentrations of lead, arsenic, PCBs and most organochlorine pesticides were detected at low concentrations which were not variable among sites, only pp'DDE was used to test correlations with immune parameters. No significant correlations were found between pp'DDE and any immune parameter, organ or body mass (Table 4).

Body and Organ Masses

There were no significant differences in body or organ masses between sexes or sibling chicks within sprayed and non-sprayed groups. Therefore the mean of the results of sibling chicks from each nest was used in the statistical analysis. There were no significant differences in body mass of 16-day old chicks among sites or between spray treatments (Table 5). Liver mass was not significantly different between spray groups but was significant among sites. Liver: body mass showed no differences among sites or between spray treatment groups. Liver masses

were not different among non-sprayed sites but were significantly different among sprayed sites ($H = 9.8$, $p = 0.008$). Mean liver mass at Orchard site 1 of 0.9430 ± 0.1200g was significantly higher than mean liver mass in birds from Orchard site 4 whose mean mass was 0.7370 ± 0.1050g.

Bursa and thymus mass, thymus: body mass were all significantly and negatively correlated with collection date (Table 4). Comparison of those parameters among spray treatments and sites, with collection date as a covariate, found significantly higher bursal mass ($F^{[1,4]} = 9.1$; $p = 0.005$) in the non-sprayed group but no differences among orchard sites or among non-sprayed sites.

Bursa: body mass was negatively correlated with collection date and the number of individual chemical applications during chick-rearing (Table 4). ANCOVA, adjusted for both covariates, showed no differences between spray treatments or among sites for bursa: body mass. Collection date and number of sprays on chicks were both significant and negative correlates with bursa: body mass ($Y = -2.48 - 0.45$ (spray) -0.47 (collection date); $R^2 = 0.32$, $p = 0.001$). Spleen mass and spleen: body mass were not significantly different among spray treatments or sites and were not correlated with collection date or spray exposures.

Histopathology

Preliminary analysis indicated that there were no significant differences between male and female chicks in any year or within any site. Therefore results of chicks were pooled within site and year. Generally histological results indicated that sprayed orchard birds showed more inflammation of the bursa and less maturation of the thymus as shown by more cellular activity in the thymic cortex and less involution, while there were few differences seen among spleens between sprayed and non-sprayed groups (Table 6). Cases of extreme histopathology were not evident for any organ but there were two cases of marked thymic involution. These were chicks from two nests at Non-sprayed site 2 in 1994. There were only two cases of bursas with 'many'

follicular cysts. One case was in 1995 in an orchard bird and the other was a bird collected in 1994 at Non-sprayed site 3.

Bursal parasites (Trematode *sp.*) were seen in only four birds in the study and these birds were sampled from Orchard 1 (n=2 in 1995; n=1 in 1994) and Non-sprayed site 3 (n=1; 1995). This may have accounted for higher bursal inflammation in sprayed birds.

Differences among sites within years

In 1994, there were no significant differences in any histological aspects of birds among non-sprayed site or among sprayed sites. In 1995, Orchard Site 2 birds had significantly lower occurrence of inflammation (0 % , n=6) of the bursal epithelium than the other sprayed sites (Orchard 1= 71.4%, n=7; Orchard 3 = 12.5%, n=7) (Table 6) while there were no significant differences for any parameter among non-sprayed sites.

Differences between years

Among four sites sampled in both years (Sprayed orchard 1, 2 and Non-sprayed sites 2, 3), only sprayed orchard 1 showed significant differences between years. At orchard 1, involution of the spleen and bursa were more pronounced in birds in 1994 compared to 1995 birds while bursal epithelium showed more inflammation in the 1995 samples.

Among all sprayed orchard birds in 1994 compared to 1995, the 1994 sprayed birds showed significantly more bursal (χ^2 = 12.6; p=0.0003) and splenic involution (χ^2 = 5.28; p=0.02). There were no differences between years in non-sprayed sites for any parameter (Table 6).

Differences in birds from sprayed orchards compared to non-sprayed sites

For 1994 sprayed sites, there were no significant differences among sites for any parameter. With the exception of bursal epithelium results, there were no differences among sprayed sites in 1995. There were no significant differences between results for any parameter among non-sprayed sites in 1994 or 1995. Therefore, non-sprayed results were pooled for comparison to the results of sprayed orchard sites in each year with the exception of bursal

epithelial evaluation. For that parameter, differences were determined among sprayed orchards and then for each sprayed orchard compared to pooled non-sprayed site results.

The Chi-square test indicated that the only significant difference among histopathological parameters was that the pooled non-sprayed site samples (1994 and 1995) had a significantly higher degree of thymic involution than birds from 1995 sprayed sites (Table 6).

Trends between histopathology results and spray exposure and collection date

For sprayed birds in both years and for non-sprayed birds in 1995, there were no relationships between collection date and the results for any histological parameter. However for 1994 results, thymic lymphocyte density was lower (χ^2 =4.5; p= 0.03)) and thymic cortex was thinner (χ^2 = 4.2; p= 0.04) as the date of collection increased with an 11-fold increase in risk of this occurring with each later collection day.

Combining sprayed and non-sprayed results for 1994 together, logistic regression further indicated that the occurrence of mild to moderate thymic involution significantly decreased as the total number of individual spray exposures per nest increased (χ^2 = 4.3, p=0.03; 70% increase of risk of occurrence with every spray exposure at the egg stage; χ^2 = 4.0, p=0.04; a 2.5-fold increase in risk of occurrence with every spray exposure during chick-rearing). Thickness of the thymic cortex increased as the number of spray exposures per nest increased during chick rearing (χ^2 = 5.1, p=0.02; a 2.5-fold increase in risk of occurrence with every spray exposure during chick-rearing). Multiple logistic regression analysis of thymic cortex thickness and collection date and spray exposure on chicks showed there was a significant probability of increasing thickness of thymic cortex with increased spray exposure (χ^2 = 9.8; a 6-fold increase in risk of occurrence with every spray exposure) but not with collection date.

Blastogenesis/ Phagocytosis /Respiratory Burst

There were no significant differences in immune parameters between sexes or sibling chicks within sprayed and non-sprayed groups. Therefore the mean of the results of immune

parameters of sibling chicks from each nest was used in the statistical analysis.

Responses to phytohemagluttinin and lipopolysaccharide were slightly higher in the orchard birds but the only blastogenic response to show differences among sites or between spray groups was that of the T and B-cell inducer, pokeweed mitogen (Table 5). There were marginally positive correlations between response to pokeweed mitogen and the individual ($R=0.34$; $p=0.04$) and mixtures ($R=0.40$; $p=0.04$) of chemicals applied during egg and chick development. There was a significantly higher response to pokeweed in the sprayed birds ($F^{[1,4]} = 6.6$; $p=0.01$) but within sprayed and non-sprayed groups there were no statistical differences (Table 5). ANCOVA adjusted for spray exposure showed no differences among spray treatments and sites.

Phagocytic response (Table 5) correlated negatively with collection date (Table 4). Comparison of spray groups and sites adjusted for collection date found no differences in any case. Similarly, net respiratory burst response was not correlated with any parameter and not different between sprayed treatments or among sites within sprayed treatment groups (Tables 3; 5).

Hematology

Collection date for all sites was negatively correlated with erythrocyte and heterophil counts, hematocrit, hemoglobin and the sum concentration of heterophil plus eosinophils (Table 4). ANCOVA to test differences among spray treatments and sites, using collection date as a covariate where necessary, revealed differences in exposure groups in mean corpuscular volume of erythrocytes which was significantly higher in the sprayed orchard birds ($F^{[1,4]} = 1119.6$; $p=0.009$) while erythrocyte numbers were significantly lower in the sprayed group ($F^{[1,4]} = 4.3$; $p=0.048$) (Table 7) suggesting anemia was present in the sprayed birds. However, those parameters did not correlate with the number of spray exposures. There were also significant differences among sites within sprayed and non-sprayed sites for erythrocyte concentration,

mean corpuscular volume, hematocrit, hemoglobin or heterophil concentrations (Table 7).

Percentage of lymphocytes in blood significantly decreased with increasing numbers of individual and mixed sprays applied per nest at the egg stage but increased with later collection dates of chicks (Table 4). ANCOVA adjusted for spray exposure and collection date found no significant differences among spray treatments or sites for percentage of lymphocytes or of heterophils. Multiple regression for each of these parameters on spray exposure and collection date found neither variable to be significantly related to percentage of lymphocytes or heterophils.

DISCUSSION

Our study is the first to examine immune parameters in wild birds exposed to pesticides in current use in a natural setting. Tree swallow chicks reared in sprayed orchards showed significantly stimulated T- and B-cell blastogenic response, and a reduction in thymic maturation which correlated with increasing exposure to pesticides. It was also found that seasonal changes, even within a period of a few weeks, were associated with changes in immune systems of these wild birds.

The finding of immunostimulation contrasts with many previous studies on occupationally pesticide-exposed humans and captive animals dosed with pesticides in which immunosuppression is the most common impact on the immune system (Vos et al., 1989; Dean and Murray, 1991; Pruett et al., 1992; Pruett, 1994).Nonetheless, stimulation of immune activity due to exposure to several types of pesticides used in these orchards is not unprecedented. For example, among the cholinesterase-inhibiting insecticides, methyl-parathion can potently stimulate lymphocyte proliferation (Pruett et al., 1989). Repeated exposure to malathion induces hypersensitivity in humans and guinea pigs, although not in mice (Milby and Epstein, 1964; Magnusson and Kligman, 1970; Cushman and Street, 1983). In other reports, B- and T-cell proliferation was significantly increased *in vivo* by malathion (Rodgers et al., 1986). Day et al.

(1995) found malathion increased spleen and thymus masses in ring-necked pheasants (*Phasianus colchicus*) but did not affect bursa however they also found significant rates of lesions in these tissues whereas we did not. Carbaryl also has a potent effect on serum immoglobulin in which titres are increased by 200-300% whereas lymphocyte proliferation is decreased in *in vitro* blastogensis assays (Andre et al., 1983). Similarly, carbachol, a cholinergic agonist, enhances the production of antibody forming cells (Pruett et al., 1992).

Exposure to organochlorine insecticides can also significantly enhance response to immune-system stimuli. Chlordane enhances the response to the mitogen concanavalin-A and to the mixed lymphocyte reaction (Johnson et al., 1986). DDT increases delayed-type hypersensitivity to bovine serum albumin (Lukic et al., 1973). Lindane increases immunoglobulin IgG_{2b} (Andre, 1983). There is also evidence that lead can induce the production of autoantibodies and promote subsequent autoimmune reactions (Waterman et al., 1994). Although concentrations of lead and most organochlorines were low and pp'DDE concentration did not correlate with any measured immune parameter in this study, the possibility of some stimulatory effect in combination with exposure to currently used pesticides cannot be discounted.

Our study also indicates that other factors influence immunology of tree swallows and need to be taken into account when interpreting results of studies on wild birds. Weather conditions may have been directly influential or have acted in combination with pesticide exposure to affect the development of the immune system and its response in these birds. During 1995, air temperatures gradually increased over time whereas in 1994 temperatures declined over the sampling period. It is well known that exposure of poultry to temperature stress (El-Halawani et al., 1973) reduces humoral (Henken et al., 1983) and cell-mediated (Regnier and Kelley, 1981) immunity, leading to altered host-resistance to infectious pathogens (Reece et al., 1992).

Alternatively, not all aspects of the immune systems were measured so there may have

been some aspect of the immune system which was suppressed while other aspects of the immune system exhibited a compensatory increase in activity. Perhaps this partially accounts for our finding of a decline in phagocytic response in tree swallow chicks over the study period while other responses such as blastogenesis increased despite the lack of trends between blastogenic activity and collection date in the tree swallows. Agricultural workers and their children exposed to pesticides have shown reductions in phagocytic activity of macrophages and neutrophils (Romash, 1987) however our findings did not indicate an association between phagocytic response and pesticide exposure.

Parasite exposure can also suppress and/or stimulate the immune responses depending on the life stage of the parasite and the host (Schleifer and Mansfield, 1993). Few birds were found with bursal trematode fluke infections although only a single cross-section of tissue from each organ was examined so there is a possibility that more birds were infected with this parasite but they were not evident by this method. Trematode parasites could account for the inflammation noted in the bursas of sprayed orchard birds and the relatively higher heterophil counts in sprayed birds. Parasites might also account for the slight anemia in sprayed birds. Blood-sucking blowfly parasites (*Protocalliphora sialia*) were often found on the legs of tree swallow chicks in both sprayed and non-sprayed sites but their effects or degree of infestation were not quantified (Bishop, unpublished).Although parasites may have induced these effects, the infestations must not have been severe since we did not document any decrease in body mass suggesting that any effects on the immune system were not secondary to generalized toxicity or extreme ill health.

There seems to have been several influences involved in altering immune parameters in these birds but frequency of pesticide exposure was often correlated with immunostimulatory effects found. The mechanisms of immunostimulation due to pesticide exposure are not well understood nor are its effects on health of any organisms. The mitogen that stimulated the

highest response in tree swallows was pokeweed which induces T and B cells (Tizard, 1992). Since the activity was high among the sprayed birds, it suggests that the populations of B lymphocytes and T cells, probably T helper cells which would contribute to the B cell response, may had been previously stimulated perhaps by pesticides. Thus populations may have been higher in sprayed birds than in non-sprayed birds and hence more cells were initially present to proliferate. Another possibility is that the sprayed birds were now exhibiting a secondary response assuming they had been previously stimulated by a pesticide or other factor and the secondary response to pokeweed was much higher than the primary response occurring in the non-sprayed birds. Perhaps stimulated immune activity was induced by suppression of corticosterone which is sensitive to contaminant exposure (Hontela 1997). As for the proliferation of cells in the thymus, these results suggest delayed normal development or stimulation of thymic T cell proliferation thus delaying the appearance of a normal rate of involution of the thymus.

The consequences of immune stimulation due to pesticide exposure may be as detrimental as a decline in immune system function. Autoimmunity and hypersensitivity are possible outcomes of stimulated immune system activity. In particular increased B-cell responses are often reported in humans and in laboratory studies examining the effects of pesticide but only suggestions of possible mechanisms have been made. Humans chronically exposed to the insecticides chlordane, chlorpyrifos, or the fungicide pentachlorophenol demonstrated elevated B lymphocyte concentrations and increased rates of autoantibodies (Broughton et al., 1990; Thrasher et al., 1993).

Mechanisms suggested for the induction of autoimmunity caused by environmental contaminants involve the direct binding of a chemical to tissues which stimulate the production of autoantibodies and/or binding to a receptor on the lymphocyte that triggers a response. For example, lead directly activates B-cells (McCabe and Lawrence, 1990) and increases B-cell/T-helper cell(subset 2) interactions (McCabe and Lawrence, 1991) and induces an increase in interleukin-6 which may lead to disregulated B-cell responses and autoimmune reactions against

neural proteins (Waterman et al., 1994).Lead altered- MBP (myelin based protein) also increases T-cell response to concanavalin-A. The concern is that several diseases such as autoimmune encephalomyelitis, multiple sclerosis, and Alzheimer's disease manifest autoantibodies to neural proteins (Waterman et al., 1994). Among cholinesterase inhibitors, induction of antibody-forming cells by methyl-parathion and phenyl phosphonothioates is putatively related to the sulfur moieties in these compounds (Pruett et al., 1989) although excessive cholinergic stimulation could also cause immunotoxicity. There are reports of cholinergic receptors on lymphocytes but the reported binding affinity of these receptors is generally much lower than those in other tissues (Strom et al., 1981; Maslinski 1989). It has been suggested that the action of methyl-parathion and carbachol might be mediated by cholinergic receptors on lymphocytes or macrophages (Pruett et al., 1992). It is also suggested that OP compounds can phosphorylate some of the same proteins phosphorylated by protein kinases involved in cellular activation but this has not been formally demonstrated (Pruett et al., 1992).

Still, most studies on the immunotoxic effects of pesticides are conducted on humans operationally exposed or laboratory mammals exposed by oral gavage and such results may not be directly applicable to wild birds exposed to chemicals during embryonic and pre-fledging development. However, contaminant-induced immune stimulation appears to have detrimental effects in other vertebrate groups and this likely applies to birds as well. It is unclear which pesticide or mixtures of chemicals may have had an effect in our study since so many chemicals were sprayed and spray patterns were not consistent from year to year. Blastogenic response was only measured in 1995 but thymic development was assessed in both years. Only results of 1994 indicated significant trends in thymic development relative to spray exposure although trends in reduced thymic involution and higher cortical cell density in 1995 were consistent with 1994 results. This suggests compounds or mixtures used in both years might be involved such as individual fungicides or insecticides or dithiocarbamate/myclobutanil mixtures. It is of further concern that these effects occurred in birds that were reared inside nest boxes and somewhat

protected from direct contact with sprayed pesticides. Birds that nest in open cup nests within orchards would be even more exposed to chemical sprays. Four species of open cup-nesting bird species have shown reduced egg and chick survival in orchards sprayed with pesticides (Patnode and White, 1991; Fleutsch and Sparling, 1994).

While this study is the first to indicate that immune stimulation occurs in birds reared in sprayed orchards and that trends in pesticide exposure coincides with this state, further studies on captive wild birds in which exposure to individual and mixtures of pesticides are controlled are necessary if pesticide involvement is to be investigated conclusively.

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Table 1. Mean values for pp'DDE and PCB residues in tree swallow eggs, and EROD activity in livers of 16-day old tree swallow chicks from four sprayed apple orchards and three non-sprayed sites in southern Ontario (1994-1997)

SITE	% Lipid in Eggs	pp'DDE (ug/g)	Sum PCB (ug/g)	Year of Egg Collection	No. Nests Sampled for eggs	EROD Activity * (pg/min/mg protein	No. Nests Sampled for livers
Orchard 1	8.08	2.29	0.37	1994/1995	10	23.6 (15.3)	7
Orchard 2	5.03	1.49	0.61	1994/1995/1997	14	24.5 (25.5)	6
Orchard 3	7.07	0.38	0.27	1994	6	34.9 (30.5)	9
Orchard 4	10.70	0.66	0.4	1994/1995	4	NM	NM
Non-Sprayed Site 1	7.03	0.36	0.37	1997	4	24.3 (32.1)	4
Non-Sprayed Site 2	8.80	0.60	0.27	1994	10	30.5 (24.5)	8
Non-Sprayed Site 3	9.21	2.23	0.49	1994/ 1995	6	10.7 (16.3)	3

N = number of nests sampled; 1 chick per nest for EROD Activity; 1 egg per nest for organochlorine residues

NM = not measured

* Mean (standard deviation) of EROD activity determined in 1995. No significant differences were found among sites for EROD Activity

Table 2. Pesticides sprayed alone or as mixtures in four apple orchards in southern Ontario (1994; 1995) and their application rates

Active Chemical	Chemical Activity	Trade Name	Type of Chemical
Metiram	Fungicide	Polyram	EBDC
Mancozeb	Fungicide	Dithane DG, M-	FBDC
Myclobutanil	Fungicide	Nova 40 W	EBI
Captan	Fungicide	Maestro	Phthalate
Azinphos-methyl	Insecticide	Guthion 50%	Che Inhibitor
Diazinon	Insecticide	Imidan	Che Inhibitor
Carbaryl	Insecticide & Growth Regulator	Sevin 50% WP	Che Inhibitor & Growth Regulator
Deltamethrin	Insecticide	Decis 5 EC	Synthetic Pyrethroid
Cypermethrin	Insecticide	Ripcord 400 E	Synthetic Pyrethroid
Endosulfan	Insecticide	Thiodan	Organochlorine
Clofentezine	Acaricide	Apollo	tetrazine
Chinomethionat	Acaricide & Fungicide	Morestan 25% WP	Quinoxaline
Oil	Insecticide & Acaricide	Superior Oil	Petroleum Hydrocarbon
Glyphosate	Herbicide	Round Up	Herbicide
NAA	Growth Regulator	NAA	Growth Regulator
Zinc (Zn 50)	Nutrient	Zinc (Zn 50)	Nutrient
Boron	Nutrient	Boron	Nutrient
Calcium	Nutrient	Calcium	Nutrient
Nitrogen	Nutrient	Nitrogen	Nutrient
1,2-dihydro-3,6-perdazinedione	Growth Regulator	Sorbatran	Growth Regulator

Table 2 (CONTINUED). Pesticides sprayed alone or as mixtures in four apple orchards in southern Ontario (1994; 1995) and their application rates

Active Chemical	Year Applied	Technical Formulation Application Rate per Hectare *
Metiram	94/95	6.0 kg
Mancozeb	94/95	3.0-6.0 kg
Myclobutanil	94/95	340 g
Captan	94	4 kg
Azinphos-methyl	94/95	2.0 kg
Diazinon	94/95	3.75 kg
Carbaryl	94/95	2.0-3.0 kg
Deltamethrin	94/95	250 ml
Cypermethrin	94/95	250 ml
Endosulfan	94	4 kg
Clofentezine	95	300 ml
Chinomethionat	94	2.25 kg
Oil	95	20 L /1000 L tank water
Glyphosate	94/95	0.8 - 1.2 kg
NAA	94/95	75-100 ppm
Zinc (Zn 50)	95	3.6 kg / 1000 L tank water
Boron	94/95	0.41 L
Calcium	95	5.0 L
Nitrogen	95	5.0 L / 1000 L tank water
1,2-dihydro-3,6-perdazinedione	95	4.5 L

* Application Rate per Hectare = Based on spray schedules provided by farmers. These rates were used in individual applications and in mixtures of chemicals.

Table 2 (CONTINUED). Pesticides sprayed alone or as mixtures in four apple orchards in southern Ontario (1994; 1995) and their application rates

Mixtures Applied **	Year Applied
Polyram/Nova	94/95
Dithane/Nova	94/95
Dithane/Nova/Imidan	94
Dithane/Apollo/Sniper /Sevin/Calcium	95
Nova/Ripcord	94/95
Dithane/Ripcord/Boron	95
Dithane/Boron	95
Zinc/Polyram	95
Nova/Dithane/Foliar Nitrog	95
Dithane/Sevin	95
Dithane/ Guthion	94
Morestan/Ripcord/ Nova	94
Maestro/Thiodan/Guthion	94

** Mixtures Applied = application rate was as described for individual chemicals listed in this table.

Table 3. Mean (Standard Deviation), maximum and minimum number of chemical applications per nest during egg and chick stages of tree swallows in four sprayed apple orchards in southern Ontario (1994- 1995)

		No. Individual Chemical Applications: Eggs 1994	Eggs 1995	Chick-rearing 1994	Chick-rearing 1995	Sum total of Individual Chemical Applications during Egg stage and Chick-rearing 1994	1995	N 1994	N 1995
Orchard									
1	mean	4.0 A	4.9 A	2.4 A	3.2 A	6.0 A	8.0 B	7	7
	(St.Dev.)	(0.8)	(1.1)	(0.8)	(1.3)	(1.2)	(1.5)		
	min.; max.	3 ; 5	4 ; 7	1 ; 3	2 ; 6	4 ; 7	7 ; 11		
2	mean	4.4 A	4.5 A	3.9 B	3.8 A	8.3 B	8.3 B	10	7
	(St.Dev.)	(0.9)	(1.8)	(0.7)	(0.4)	(1.2)	(1.8)		
	min.; max.	3 ; 6	1 ; 6	3 ; 5	3 ; 4	6 ; 10	5 ;10		
3	mean	NS	2.2 B	NS	3.4 A	NS	5.7 A	NS	9
	(St.Dev.)		(1.5)		(0.5)		(1.1)		
	min.; max.		1 ; 4		3 ; 4		4 ; 7		
4	mean	3.8 A	NS	2.2 A	NS	6.0 A	NS	6	NS
	(St.Dev.)	(1.3)		(1.0)		(0.9)			
	min.; max.	2 ; 6		1 ; 3		5 ; 7			

NS = not sampled in that year
N = number of nests sampled
Different letters (A, B etc.) among sites within year and parameter indicate significant differences $p < 0.05$

Table 3 (CONTINUED). Mean (Standard Deviation), maximum and minimum number of chemical applications per nest during egg and chick stages of tree swallows in four sprayed apple orchards in southern Ontario (1994- 1995)

		No. Mixed Spray Events: No. Individual Chemical Appli				Sum Total of Mixed Spray Applications during Egg stage and Chick-rearing		N	N
		Eggs	Eggs	Chick-rearing	Chick-rearing				
		1994	1995	1994	1995	1994	1995	1994	1995
Orchard									
1	mean	2.1 A	2.6 A	0.9 A	1.0 A	2.1 A	2.6 A	7	7
	(St.Dev.)	(0.4)	(0.5)	(0.4)	(0.6)	(0.4)	(0.5)		
	min.; max.	2 ; 3	2 ; 3	0 ; 1	0 ; 2	2 ; 3	2 ; 3		
2	mean	1.3 A	1.0 A	1.0 A	0.7 A	1.3 A	1.0 A	10	7
	(St.Dev.)	(0.5)	(0)	(0)	(0.5)	(0.5)	(0)		
	min.; max.	1 ; 2	1 ; 1	1 ; 1	0 ; 1	1 ; 2	1 ; 1		
3	mean	NS	1.1 A	NS	0.7 A	NS	1.1 A	NS	9
	(St.Dev.)		(0.3)		(0.7)		(0.3)		
	min.; max.		1 ; 2		0 ; 2		1 ; 2		
4	mean	2.0 A	NS	0.5 A	NS	2.0 A	NS	6	NS
	(St.Dev.)	(0)		(0.5)		(0)			
	min.; max.	2 ; 2		0 ; 1		2 ; 2			

NS = not sampled in that year
N = number of nests sampled
Different letters (A, B etc.) among sites within year and parameter indicate significant differences $p < 0.05$

Table 4. Correlation coefficients (R value) for body and organ weights, immune responses, and hematological parameters in 16-day old tree swallows from three sprayed apple orchards and three non-sprayed sites versus collection dates and spray exposures per nests in southern Ontario (1994; 1995)

Variable	Year	Collection Date for 16-day old chick (1995)		
		Sprayed Sites	Non-sprayed Sites	All Sites Combined
Organ and Body Mass **:				
Body Mass	1995	-0.41	-0.23	-0.10
Bursa Mass	1995	**-0.67 ***	**-0.78 ***	**-0.50 ***
Spleen Mass	1995	0.29	-0.29	-0.01
Thymus Mass	1995	-0.41	-0.46	-0.40
Liver Mass	1995	-0.03	0.09	0.04
Liver:Body Mass	1995	0.19	0.09	0.07
Bursa:Body Mass	1995	**-0.65 ***	**-0.65 ***	**-0.40 ***
Spleen:Body Mass	1995	0.36	-0.22	-0.06
Thymus:Body Mass	1995	-0.18	-0.46	**-0.36 ***
Phagocytosis **	1995	**-0.73 ***	-0.52	**-0.45 ***
Net Respiratory Burst **	1995	0.35	-0.17	-0.0086
Blastogenesis **:				
Concanavalin A	1995	-0.39	-0.06	-0.15
Phytohemagglutinin	1995	-0.21	-0.04	-0.06
Lipopolysaccharide	1995	-0.003	0.24	0.15
Pokeweed	1995	-0.13	-0.02	-0.11
Hematology *:**				
Erythrocyte Concentration	1995	**-0.56 ***	-0.90	**-0.7 ***
Hemoglobin	1995	-0.42	-0.89	**-0.69 ***
Hematocrit	1995	**-0.71 ***	-0.84	**-0.77 ***
Mean Corpuscular Volume	1995	-0.28	0.05	0.002
% Heterophils	1995	**-0.62 ***	**-0.51 ***	**-0.52 ***
Heterophil Concentration	1995	**-0.83 ***	**-0.92 ***	**-0.86 ***
% Lymphocytes	1995	**0.65 ***	0.46	**0.52 ***
Lymphocyte Concentration	1995	-0.005	-0.27	-0.18
% Monocytes	1995	0.33	-0.07	0.2
Monocyte Concentration	1995	0.16	0.25	0.015
% Basophils	1995	0.13	0.33	0.11
Basophil Concentration	1995	0.07	-0.30	-0.14
Heterophil + Eosinophil Concentration	1995	**-0.88 ***	**-0.89 ***	**-0.88 ***
EROD Activity ***	1995	-0.08	-0.27	-0.19
Collection Date ***	1994	-	-	-
	1995	-	-	-

* p <= 0.01; ** For these variables, R Value represents Pearson Product-Moment Correlation Coefficient; - = Not Applicable

*** For these variables, R Value represents Spearman Rank Correlation Coefficient

Table 4 (CONTINUED). Correlation coefficients (R value) for body and organ weights, immune responses, and hematological parameters in 16-day old tree swallows from three sprayed apple orchards and three non-sprayed sites versus collection dates and spray exposures per nests in southern Ontario (1994; 1995)

Variable	Year	Number of Individual Chemicals sprayed during:	
		Egg Stage	Chick-rearing
Organ and Body Mass **:			
Body Mass	1995	0.07	0.06
Bursa Mass	1995	0.008	-0.01
Spleen Mass	1995	-0.0052	-0.04
Thymus Mass	1995	0.10	0.06
Liver Mass	1995	0.17	0.17
Liver:Body Mass	1995	0.17	0.16
Bursa:Body Mass	1995	-0.01	**-0.37 ***
Spleen:Body Mass	1995	0.0028	0.10
Thymus:Body Mass	1995	0.15	0.07
Phagocytosis **	1995	0.52	0.51
Net Respiratory Burst **	1995	0.26	0.25
Blastogenesis **:			
Concanavalin A	1995	0.19	0.17
Phytohemagglutinin	1995	0.16	0.13
Lipopolysaccharide	1995	0.03	0.13
Pokeweed	1995	0.29	0.38
Hematology *:**			
Erythrocyte Concentration	1995	0.17	0.17
Hemoglobin	1995	-0.11	-0.21
Hematocrit	1995	0.024	-0.26
Mean Corpuscular Volume	1995	0.29	0.26
% Heterophils	1995	0.27	0.004
Heterophil Concentration	1995	0.25	0.21
% Lymphocytes	1995	**-0.31 ***	-0.02
Lymphocyte Concentration	1995	-0.15	-0.11
% Monocytes	1995	0.06	0.18
Monocyte Concentration	1995	0.11	0.15
% Basophils	1995	-0.23	-0.23
Basophil Concentration	1995	-0.07	-0.04
Heterophil + Eosinophil Concent	1995	0.24	0.01
EROD Activity ***	1995	-0.07	-0.10
Collection Date ***	1994	-0.28	0.24
	1995	**-0.73 ***	**0.58 ***

* p <= 0.01; ** For these variables, R Value represents Pearson Product-Moment Correlation Coefficient; - = Not Applicable

*** For these variables, R Value represents Spearman Rank Correlation Coefficient

Table 4 (CONTINUED). Correlation coefficients (R value) for body and organ weights, immune responses, and hematological parameters in 16-day old tree swallows from three sprayed apple orchards and three non-sprayed sites versus collection dates and spray exposures per nests in southern Ontario (1994; 1995)

Variable	Year	Number of Mixtures of Chemicals sprayed during:		pp'DDE conc	EROD Activity
		Egg Stage	Chick-rearing	Eggs	Liver
Organ and Body Mass **:					
Body Mass	1995	0.23	0.41	0.33	-
Bursa Mass	1995	0.08	0.19	-0.28	-
Spleen Mass	1995	0.09	0.28	0.11	-
Thymus Mass	1995	0.11	0.20	-0.16	-
Liver Mass	1995	0.19	0.22	0.25	-0.27
Liver:Body Mass	1995	0.18	0.19	0.23	-0.27
Bursa:Body Mass	1995	-0.04	0.05	-0.14	-
Spleen:Body Mass	1995	-0.04	0.10	0.17	-
Thymus:Body Mass	1995	-0.06	0.26	-0.13	-
Phagocytosis **	1995	0.51	0.47	-0.12	-0.17
Net Respiratory Burst **	1995	0.26	0.26	-0.03	0.01
Blastogenesis **:					
Concanavalin A	1995	0.22	0.27	0.05	0.17
Phytohemagglutinin	1995	0.26	0.21	-0.04	0.03
Lipopolysaccharide	1995	0.13	0.05	0.09	0.20
Pokeweed	1995	0.33	0.40	0.13	0.10
Hematology *:**					
Erythrocyte Concentration	1995	0.15	0.05	0.002	-0.18
Hemoglobin	1995	-0.03	0.04	-0.023	-0.08
Hematocrit	1995	-0.001	-0.027	0.01	-0.05
Mean Corpuscular Volume	1995	0.18	0.05	0.013	0.02
% Heterophils	1995	0.19	0.1	0.09	-0.10
Heterophil Concentration	1995	0.22	0.13	0.06	-0.10
% Lymphocytes	1995	-0.21	-0.14	-0.12	0.12
Lymphocyte Concentration	1995	-0.06	-0.05	-0.08	0.014
% Monocytes	1995	0.06	-0.07	0.13	0.007
Monocyte Concentration	1995	0.07	-0.12	0.15	0.002
% Basophils	1995	-0.2	-0.07	0.08	0.10
Basophil Concentration	1995	-0.07	0.01	0.15	-0.12
Heterophil + Eosinophil Concent	1995	0.18	0.09	0.03	0.007
EROD Activity ***	1995	-0.07	-0.22	0.06	1.0
Collection Date ***	1994	-0.74 *	0.30	-	-
	1995	-0.60 *	-0.28	-	-

* p <= 0.01; ** For these variables, R Value represents Pearson Product-Moment Correlation Coefficient; - = Not Applicable

*** For these variables, R Value represents Spearman Rank Correlation Coefficient

Table 5. Mean (Standard Deviation) of selected immune parameters in 16-day old tree swallow chicks from three sprayed apple orchards and three non-sprayed sites in southern Ontario (1995)

Parameter		Sprayed Orchards	Non-sprayed Sites
		N=23 nests *	N=15 nests *
Body Mass	(g)	20.5 (1.6)	20.3 (.96)
Bursa Mass	(g)	.0384 (.015) **	.0460 (.014) **
Bursa: Body Mass		.0019 (.0007)	.00234 (.0006)
Spleen Mass	(g)	.0392 (.018)	.0427 (.027)
Spleen: Body Mass		.002 (.0009)	.00215 (.001)
Thymus Mass	(g)	.0762 (.025)	.0668 (.022)
Thymus: Body Mass		.0039 (.0011)	.0037 (.001)
Liver Mass	(g)	.8320 (.136)	.7877 (.235)
Liver: Body Mass		.040 (.006)	.0391 (.011)
Phagocytosis (% of macrophage cells containing 3+ beads)	%	17.9 (.10)%	18.9 (.06)%
Net Respiratory Burst (mean FL of stimulated cells - mean FL of resting cells)	Mean Fluorescence (FL)	27.4 (8.7)%	30.4 (13.1)%
Blastogenesis :	Tritiated Thymidine uptake (cpm) in presence of :		
Concanavalin A	30 ug/ml	1323.5(1342)	2070.1(4615.4)
Phytohemagglutinin	12.5 ug/ml	4636.2(5343.8)	4327.1 (7464.6)
Lipopolysaccharide	50 ug/ml	575.8(402.3)	516.4 (488.7)
Pokeweed	12.5 ug/ml	5132.8(5655.8) ***	1579.7 (1422.1) ***

* 2 chicks per nest sampled

** Indicates significant differences between Orchard and Non-sprayed Sites ($p<=0.05$) based on ANCOVA with collection date as covariate

*** Indicates significant differences between Orchard and Non-sprayed Sites ($p<=0.05$); No adjustment for collection date

Table 6. Histopathological assessments of immune organs of 16-day old tree swallow chicks from four sprayed apple orchards and three non-sprayed sites in southern Ontario (1995)

	Sprayed Orchard 1994 N=23 nests*	Sprayed Orchard 1995 N=22 nests*	Non-Sprayed Sites 1994 & 1995 N=22 or 23 nests*
	(%)	(%)	(%)
Thymus			
Moderate to High Cortical Lymphocyte Density	74.0 A	95.4 A	72.8 A
Absent or Thin Cortex	26.1 A	22.7 A	45.5 A
Few to Many Tingible Body Macrophages	26.0 A	31.8 A	36.6 A
Presence of Heterophils	82.6 A	95.4 A	86.3 A
Overall Evaluation of Thymic Histology: Involution is Mild to Marked	47.8 A	27.2 A	68.2 B
Spleen			
T-dependent white pulp: Periarteriolar/periellipsoidal sheath Absent	30.4 A	5.3 A	13.6 A
B-dependent white pulp: Number of follicles: Few to Many present	60.9 A	66.7 A	63.4 A
Overall Evaluation of Spleen Histology:			
Involuted/Depleted or Undeveloped	63.4 A	22.2 A	54.5 A
Hyperplastic	0	0	0

Statistical differences were determined after adjustment for collection date as a covariate, where applicable.
* = 1 chick per nest sampled
For each parameter, different letters indicate significant differences between Orchard and Non-sprayed Sites.

Table 6 (CONTINUED). Histopathological assessments of immune organs of 16-day old tree swallow chicks from four sprayed apple orchards and three non-sprayed sites in southern Ontario (1995)

	Sprayed Orchard 1994 N=23 nests*	Sprayed Orchard 1995 N=22 nests*	Non-Sprayed Sites 1994 & 1995 N=22 or 23 nests*
	(%)	(%)	(%)
Bursa of Fabricius			
Epithelium			
Presence of Inflammation	81.8 A	68.1 A	40.9 A
Mucous metaplasia present	0	0	0
Squamous metaplasia present	0	0	0
Follicles			
Cellular density moderate to high	2 A	0 A	9.1 A
Occurrence of Apoptoses: Moderate to High	90.9 A	54.5 A	63.6 A
Follicular cysts: Few to Many present	100 A	95.5 A	95.4 A
Parasites Present	4.6 A	9.1 A	4.6 A
Overall Evaluation of Bursa Histology:			
Involution is mild to marked	59.0 A	4.6 A	27.2 A

Statistical differences were determined after adjustment for collection date as a covariate, where applicable.
* = 1 chick per nest sampled
For each parameter, different letters indicate significant differences between Orchard and Non-sprayed Sites.
** = For bursa N=23 nests

Table 7. Mean (Standard Deviation) of hematological parameters in blood from 16-day old tree swallow chicks from three sprayed apple orchards and three non-sprayed sites in southern Ontario (1995)

Parameter	Unit	Sprayed Orchards			Combined Orchards **
		Orchard 1 *	Orchard 2 *	Orchard 3 *	
Sample of Nests per Site	1 chick per nest	N = 2 - 7	N = 5 - 6	N = 5 - 9	N = 19 - 22
Erythrocyte Concentration	x 10E12 Cells / l	2.3 (0.3) BC	2.1(0.3) AB	2.3 (0.4) AB	2.3 (0.4) A ***
Hemoglobin	g / l	89.6 (11.2) AB	79.5 (13.0) BC	84.3 (11.8) B	84.7 (14.6) A
Hematocrit	%	33.0 (3.0) AC	37.0 (29.0) C	30.0 (4.0) C	30 (5.0) A
Mean Corpuscular Volume	fl	127.9 (4.1) B	133.9 (8.6) B	137.8 (15.4) B	133.3 (12.3) A
% Heterophils	%	74.5 (0.1) A	67.5 (0.2) A	61.5 (0.2) A	68.3 (18.0) % A
Heterophil Concentration	x 10E9 Cells / l	7.7 (5.2) A	4.0 (1.6) BC	3.2 (2.3) BC	4.7 (3.9) A
% Lymphocytes	%	16.7 (0.1) A	20.7 (0.09) A	27.7 (14.3) A	22.7 (13.2)% A
Lymphocyte Concentration	x 10E9 Cells / l	1.4 (0.8) A	1.2 (0.7) A	1.6 (1.5) A	1.4 (1.1) A
% Monocytes	%	2.0 (1.0) A	4.4 (3.5) A	6.2 (7.0) A	2.5 (4.6) A
Monocyte Concentration	x 10E9 Cells / l	0.19 (0.02) A	0.16 (0.1) A	0.22 (0.22) A	0.09 (0.15) A
% Basophils	%	0.12 (0.09) A	0.09 (0.12) A	0.13 (0.08) A	7.6 (10.2) % A
Basophil Concentration	x 10E9 Cells / l	1.1 (0.7) A	0.76 (1.2) A	0.82 (0.7) A	0.58 (0.87) A
Heterophil + Eosinophil Conce	x 10E9 Cells / l	7.7 (5.2) A	4.0 (1.6) BC	3.3 (2.3) BC	6.7 (4.1) A

* Different letters indicate significant differences among all sites (orchard plus control sites) after adjustment for the covariate of collection date, where necessary.
** Different letters indicate significant differences between treatment groups (ie. combined orchard results versus combined control site results) after adjustment for the covariate of collection date, where necessary.
*** LSD Means: 2.3 (0.35) x 10E12 cells/l Orchards: 2.5 (0.7) x 10E12 cells/l Non-Sprayed Sites
**** Not included in among site analysis due to small sample size (N=1); Note: 10E9 = 1000000000 and 10E12 = 1000000000000

Table 7 (CONTINUED). Mean (Standard Deviation) of hematological parameters in blood from 16-day old tree swallow chicks from three sprayed apple orchards and three non-sprayed sites in southern Ontario (1995)

Parameter	Unit	Non-sprayed Sites			
		Site 1 *	Site 2 *	Site 3 *	Combined Control Sites **
Sample of Nests per Site	1 chick per nest	N = 2 - 4	N = 4 - 8	N = 1 - 3	N = 14 - 15
Erythrocyte Concentration	x 10E12 Cells / l	2.2 (0.3) AB	2.8 (0.6) BC	1.8 (0.6) A	2.6 (0.7) B ***
Hemoglobin	g / l	77.5 (11.4) BC	100.6 (21.7) A	63 (18.0) C	88.6 (24.6) A
Hematocrit	%	29.0 (2.0) CB	34.7 (5.4) A	48.4 (68.2) B	31.0 (6.0) A
Mean Corpuscular Volume	fl	132.6 (13.8) A	63.8 (64.1) A	131.1 (14.1) A	93.1 (62.0) B
% Heterophils	%	53.0 (24.3) B	70.5 (17.7) A	44.0 (8.7) B	60.4 (22.8) % A
Heterophil Concentration	x 10E9 Cells / l	3.4 (2.3) BC	5.3 (2.7) B	1.1 (0.16) C	3.9 (2.9) A
% Lymphocytes	%	40.2 (24.0) A	21.5 (14.7) A	43.5 (7.9) A	30.9 (20.3) % A
Lymphocyte Concentration	x 10E9 Cells / l	2.3 (1.6) A	2.1 (2.4) A	1.6 (0.40) A	1.9 (2.1) A
% Monocytes	%	1.5 (.5) A	1.8 (0.8) A	3.5 (0) ****	.78 (1.1) % A
Monocyte Concentration	x 10E9 Cells / l	0.15 (0.1) A	0.15 (.16) A	0.11 (0) ****	0.06 (0.11) A
% Basophils	%	6.9 (3.2) A	7.4 (2.8) A	28.7 (23.2) A	10 (12.4) % A
Basophil Concentration	x 10E9 Cells / l	0.38 (0.21) A	.64 (0.6) A	0.71 (0.54) A	0.5 (0.5) A
Heterophil + Eosinophil Conc.	x 10E9 Cells / l	3.4 (2.3) BC	5.3 (2.7) B	1.1 (0.15) C	3.9 (2.9) A

* Different letters indicate significant differences among all sites (orchard plus control sites) after adjustment for the covariate of collection date, where necessary.

** Different letters indicate significant differences between treatment groups (ie. combined orchard results versus combined control site results) after adjustment for the covariate of collection date, where necessary.

*** LSD Means: 2.3 (0.35) x 10E12 cells/l Orchards: 2.5 (0.7) x 10E12 cells/l Non-Sprayed Sites

**** Not included in among site analysis due to small sample size (N=1); Note: 10E9 = 1000000000 and 10E12 = 1000000000000

Chapter Two.

Health of Tree Swallows *(Tachycineta bicolor)* Nesting in Pesticide-Sprayed Apple Orchards in Ontario, Canada.

Sex and Thyroid Hormone Concentrations and Testes Development

Health of Tree Swallows *(Tachycineta bicolor)* Nesting in Pesticide-Sprayed Apple Orchards in Ontario, Canada

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ABSTRACT

To investigate the effects of pesticides on wild birds, sex (17 β-estradiol; testosterone) and thyroid (tri-iodo-thryonine(T3)) hormone concentrations, body and testes masses were measured and evaluated the development of testes were evaluated in wild tree swallows (*Tachycineta bicolor*) nesting in four sprayed apple orchards and three non-sprayed sites in southern Ontario, Canada in 1995-1996. In orchards, birds were exposed to as many as 11 individual spray events and five sprays of mixtures of chemicals. Residues of organochlorine pesticides, PCBs, lead, and arsenic concentrations were low and not variable among sites except pp'DDE concentrations which ranged from 0.36 to 2.23 ug/g wet wt. in eggs. These persistent compounds were not correlated with any endocrine response measured in tree swallows.

In 16-day old male tree swallow chicks, body mass, and concentrations of 17 β-estradiol (estradiol), testosterone, T3 in plasma showed no significant differences between sprayed and non-sprayed groups and among sites within those groups. However, T3 concentrations were slightly elevated in the sprayed group compared to the non-sprayed group and there was a significant and positive correlation between T3 and the number of mixtures of sprays applied during egg incubation through chick-rearing.

In 16-day old female chicks, there were no significant differences among spray treatments or sites and no correlations with spray exposure for testosterone, estradiol or T3 in plasma. Body mass was correlated positively with T3 and negatively with estradiol but showed no differences among spray exposure groups or sites.

Histology of testes of 16-day old male chicks indicated there were no significant differences among sprayed and non-sprayed birds in testes mass, area, or diameter, or the presence of Leydig cells in the interstitium, the distribution of the sertoli cells, or the occurrence of heterophils in the testicular interstitium. For the percentage of spermatogonia present on the basement membrane, there were significant differences among sites but these differences were not specifically associated with spray exposure. However, there was a marginally significant trend between increasing occurrence of a disrupted sertoli cell population on the seminiferous tubular basement membranes as the number of mixtures of pesticides sprayed during chick-rearing increased.

In adult male and female parent tree swallows, there were no differences in hormone concentrations between birds from sprayed and non-sprayed sites. Nor were there any significant correlations between the concentration of any hormone and collection date, body mass or any type of spray exposure for adults.

The correlations between increasing pesticide exposure and abnormal thyroid hormone and testes development in male chicks indicates further reductions of pesticide use in orchards may benefit the health of birds that nest there. However, it is unclear which of these pesticides or sprays mixtures are responsible for these effects and this needs to be examined in future studies.

INTRODUCTION

Many chemicals released into the environment in the past and present can alter endocrine function and one of the most prevalent concerns about the effect of environmental contamination is possible damage to the reproductive system of wildlife and humans (Colborn and Clement, 1992). Wildlife populations exposed to high concentrations of chlorinated hydrocarbons have experienced embryotoxicity and teratogenesis in the Great Lakes basin and elsewhere (Geisy et al 1994). Hormonal imbalances and abnormal sexual morphology have recently been found in alligators exposed to DDT metabolites and other pesticides in Lake Apopka, Florida (Guillette et al 1994;1996). Effects of contaminants on thyroid function are also well documented, particularly in birds (Peakall et al 1992). The potential effects on hormones and secondary sex characteristics of less persistent compounds such as cholinesterase-inhibiting pesticides, and fungicides commonly used today have been less thoroughly described although *in vitro* and captive-dosing studies have indicated that some of these chemicals have the potential to cause endocrine dysfunction (Hess et al 1991; Laisi et al 1985; Maitra and Sarkar, 1993).

In apple orchards, birds are exposed to in-use chemicals as well as residues of historically used chemicals such as organochlorine pesticides, and lead arsenate (Blus et al 1987; Eisler 1988). As part of a study to examine the health of birds living in orchards, several endocrine endpoints were examined including sex and thyroid hormone levels and the histological development of testes in wild tree swallows nesting in sprayed apple orchards and non-sprayed sites. The hypothesis was that the endocrine endpoints would show significant differences among sprayed and non-sprayed tree swallow adults and chicks.

METHODS

Study Area

All sites were located in the Great Lakes basin in southern Ontario, Canada (~ latitude 43°15 'N/ longitude 80°20' W). This study was conducted in 1995-1996 in the same four orchards

and three non-sprayed (control) sites used in 1994-1995 study in Bishop et al (this volume). The study area and establishment of nest boxes for tree swallows in sprayed orchards and non-sprayed sites are described in that report.

Sample Design and Weather Conditions

The number of nests sampled per site varied from three to eight among study sites in each year of the study. In total, sample size was 32 nests in 1995 and 16 nests in 1996 from sprayed orchards. From non-sprayed sites, sample size was 29 nests in 1995 and 14 nests in 1996.

In 1995, whole blood was collected and plasma was analyzed for hormones from two 16 day old chicks per nest and from male and female adult birds of the same nests from which chicks were sampled as well as some others. Because adults could not be trapped anywhere but at the nest, dates were arbitrarily chosen during the nesting period when parent birds were easiest to catch. For adult females, blood was collected at the ninth or tenth day of egg incubation in each nest. For males, blood was collected on the six or seventh day after egg-hatching. In 1996, histological assessment of testes was performed on one male chick from each nest from three sprayed orchards and three non-sprayed sites.

Body Mass, Blood and Tissue Collection

All birds were handled according to the Canadian Council on Animal Care (Olfert et al., 1993) and procedures were approved by the Canadian Wildlife Service Animal Care Committee. Mass of birds was measured in the field with a 50 (+/- 0.5)g Pesola scale. Blood was collected from birds by jugular venipuncture within five minutes of removing the bird from the nest. Samples were collected between 0830 and 1039 hrs each day. The procedures described in Bishop et al (this volume) were followed except that the maximum blood volume sampled per bird was 15% of total blood volume. In the field, blood was spun in a microhematocrit centrifuge for five minutes and stored on ice for 2-4 hours. Afterwards, plasma samples were placed in -

20°C until analysis for hormones.

Testes were removed in the field from birds killed by carbon dioxide asphyxiation. After dissection, testes were immediately placed in 10% buffered formalin for later histological examination. Carcasses were stored on ice and then placed at -20°C until analysis for lead and arsenic. All samples were coded with generic identification numbers to facilitate 'blind' laboratory analyses.

Hormone Analyses

17β-Estradiol and Testosterone

Testosterone and 17β-estradiol were measured in duplicate using radioimmunoassay (Van Der Kraak et al 1984; Van Der Kraak et al 1990) on plasma following ether extraction of 25 ul plasma. Detection limit was 312 pg/ml plasma for 17β-estradiol and testosterone.

Triiodo-L-thyronine (T3)

Triiodo-L-thyronine (T3) concentrations were assayed in undiluted plasma using an antibody-coated I^{125} radioimmunoassay kit (ICN, No. 07-292102) (Hontela et al., 1995). Analysis for hormones was performed on 100 ul sample of plasma. The assay was performed on two replicate 100 ul sub-samples and results were a mean of those results. Detection limit was 0.15 ng/ml.

Histological Evaluation: Testes

Paired testes mass was determined to the nearest 1/100 g and embedded in paraffin wax. Serial sections (5 µm) were cut and stained with hematoxylin and eosin, and examined under a 100 power light microscope.

For each nestling, the area (mm^2) and diameter (mm) of the largest testis was determined and the total number of seminiferous tubules was counted in its cross-section. The sertoli cells were assessed based upon whether a) the cells formed a broken layer on or above the basement membrane and spermatogonia or b) cells formed a contiguous layer above the

basement membrane. The spermatogonia were evaluated as a) comprising 20-50% of the population on the basement membrane or b) comprising greater than 50% of the cells on the basement membrane. Leydig cells were evaluated as either rare or clearly identifiable in the interstitium. The presence of heterophils was described in the testicular cross-section as a) 0-2 heterophils present b) 3-6 heterophils present c) or a concentrated focus of heterophils in the interstitium.

Exposure

Chemical Applications in 1995 and 1996

In all apple orchards, multiple types of pesticides and spray events occurred during the approximately 35 day period (Peck and James, 1987) from the first egg oviposited per nest through egg incubation, to the day before hatching (defined this as the 'egg stage'), from hatching and development to 16 day-old chicks (chick-rearing) in each orchard. Each farmer provided the exact date of spray, name of the chemical and its application rate for each orchard (Table 1) which were consistent with the recommendations published by the provincial government (OMAFR, 1994;1996).

Pesticides in were applied with an air-blast sprayer by the orchard owners who are licenced pesticide applicators. The chemicals were applied according to the farmer's choice of spray schedule which depended on pest cycles in the orchard and advisories from the Ontario Ministry of Agriculture and Rural Affairs (OMAFR) 'agri-phone' and Fruit Production Recommendations (OMAFR, 1996). Therefore these study sites were generally representative of chemical application methods and schedules used in apple orchards in Ontario.

Because pesticides can diffuse through avian eggshells (Hoffman, 1990) exposure to the embryo could occur as soon as eggs are laid. Therefore, the number of times each nest was exposed to a chemical application during each of the egg and chick-rearing stages was determined. For those periods, the number of occasions each nest was exposed to a) two or

more chemicals applied as a tank mixture or b) exposed to two or more chemicals sprayed sequentially on the same day ie. mixtures or mixed spray events, was summed. The number of individual chemical spray events and mixed spray events to which each adult was exposed for the period of the seven days prior to the first egg laid in each nest through to the date of blood collection was also summed.

Chlorinated Hydrocarbon Analyses

Methods for measuring organochlorine pesticides (OCs), chlorobenzenes and total PCB congeners (PCBs) are based on those of Peakall et al. (1986) and Bishop et al. (1996) and described in Bishop et al (this volume).

Lead and Arsenic Analyses

The livers from male chicks collected for testes evaluation were analyzed for lead and arsenic. Metals analyses were carried out on one chick from each nest. Two to six nests per study site were sampled. Livers from each site were pooled and analyzed as a single sample.

For analysis, liver samples were weighed and placed in plastic, acid washed test tubes, freeze-dried and their wet and dry weights were recorded (approx. 0.5g wet weight). To each test tube 0.5 ml of deionized water then 0.5 ml of HNO_3 (70%) per 0.1g dry weight were added. Samples then sat overnight at room temperature. The following day samples were heated, loosely capped, at 100° C in dry baths for approximately six hours. The samples were left to cool overnight, then the volumes were adjusted to 4 ml with deionized water.

Lead and arsenic were analyzed by graphite furnace spectrometry (GFASS) using a Perkin Elmer 3030b equipped with Deuterium Background Corrector, HGA-300 Graphite Furnace and AS-40 autosampler. For lead, determinations were made with the calibration performed versus acid standards (10% nitric acid). Ammonium phosphate was used as a 'matrix modifier', argon was used as a purge gas. Sensitivity was 12 pg. The detection limit was 0.06 ug/g dry weight for 0.2 g sample. Recovery of the reference material was 95-104 %. For arsenic,

determinations were made with the method of standard addition and were based on measuring a peak area. Nickel was use as a matrix modifer, argon was the purge gas. Detection limit was 0.2 ug/g dry weight for 0.2 g sample. Recovery of reference material was 96.7-99.2%.

Statistical Analysis

In this study, data were log-transformed, where necessary, to meet normality and homogeneity of variance requirements for parametric analysis, otherwise, non-parametric tests were used. All statistical analyses were considered significant at $p \leq 0.05$ (Sokal and Rohlf, 1981) except correlations between collection date, spray exposures and endocrine parameter where $p=0.01$ was used due to the large number of correlations performed. All correlation results found significant were examined by scatterplots to confirm credible gradients in results occurred. All data were analyzed using STATISTICA for Windows (Statsoft, 1997).

For histology results, nested analysis of variance (ANOVA)for unequal sample sizes or the Kruskal-Wallis test and Tukey's test for unequal sample sizes were used to compare between exposure groups and among sites within those groups for testes diameter, mass, area, and total number of seminiferous tubules (Sokal and Rohlf 1981). Results for sertoli cells, spermatogonia, heterophils and Leydig cells were compared between sprayed and non-sprayed birds using the Chi-square test with Yates' correction (Sokal and Rohlf 1981).

Nested ANOVA for unequal sample sizes or Kruskal-Wallis tests and Tukey's test for unequal sample sizes were used to compare body mass and hormone concentrations between spray-exposure groups and among individual sites. Where endpoints correlated with collection date or other parameters, these variables were incorporated as covariates in an Analysis of Covariance (ANCOVA) (Sokal and Rohlf, 1981).

The number of individual and mixtures of chemicals applied during each nest's development in each orchard were compared among orchards using the Kruskal-Wallis test and Tukey's test for unequal sample sizes. The same analysis was performed on the number of

sprays and mixtures of chemicals applied during the period of seven days prior to egg laying to the date of sampling of adult male and female birds.

The egg and tissue concentrations of organochlorine pesticides, PCBs, lead and arsenic present and a statistical comparison among groups are reported in Bishop et al (this volume). Those data were used to examine trends between residue levels and endocrine endpoints.

Because 16-day old chicks were collected during a 5-10 day period within each site, spray exposures varied among nests during egg and chick development in orchards. Nest initiation dates also varied among nests making exposure to pesticides differ among adult birds. It was determined if factors other than spray exposure coincided with trends in endocrine response by examining correlations between collection date (ie. progression of the season), body mass and each endocrine endpoint. It was also determined if weather conditions, as represented by air temperature on the collection date, correlated with sample collection dates.

Product-moment or Spearman rank correlations, or logistic regressions (Sokal and Rohlf, 1981; Fox, 1984) were performed to examine the trends between the number and type of spray exposures per nest or persistent contaminant residues in tissues, versus histological results, hormone concentrations and testes results. Where collection date or body mass were significant, and multicolinearity with spray exposure variables did not occur, multiple regression analysis was performed to determine the significance of all variables (Sokal and Rohlf, 1981).

RESULTS

Sample Date and Weather conditions

There was a weak but significant and positive correlation (Spearman Rank $R^2 = 0.15$; $p=0.04$) between collection date and maximum air temperatures (Environment Canada, 1995; 1996) on the day before sampling and on the day of sampling of adult female birds in 1995. No correlations for dates of sampling of tree swallow chicks for histology of testes in 1996 or blood sampling of adult males in 1995 were found. However, in 1995, from the same nests that tree

swallow chicks were sampled to determine hormone levels, other chicks were also sampled on the same day to examine immune parameters in a related study (Bishop et al, this volume). In Bishop et al (this volume) they report that air temperatures increased significantly during the sample period in early June to July in 1995.

Chemical Exposure

Chemical Applications in 1995 and 1996

Chemicals Applied

Between years, the type of chemicals and, in particular, the mixtures applied were quite different (Table 1) whereas the number of exposures per nest to chemicals was similar (Table 2a;b). There were some similarities among the types of chemicals applied in each year with at least one type of organophosphorus, carbamate, and synthetic pyrethroid insecticide and one ethylene-bis-dithiocarbamate fungicide and myclobutanil fungicide being applied in both years.

In 1995, the number of individual chemical applications per nest during chick development was positively correlated with blood sample collection date whereas exposure to individual sprays and to mixtures per nest during egg development were negatively correlated with sample collection date for 16-day-old chicks (Bishop et al., this volume). For adult males, spray exposures were not correlated with blood sampling date. For adult females, the number of individual chemical exposures was negatively correlated with blood sampling date (R^2= 0.34; p=0.001). In 1996, collection date was not correlated with individual chemical or mixed spray exposures on eggs or chicks.

Applications during egg and chick development

In 1995 and 1996, the number of chemical exposures was similar during egg stage and chick-rearing. In both years, the number of chemical applications during embryonic development varied from one to seven and during chick-rearing from a minimum of two or three to six or seven sprays. Likewise, nests were exposed to between zero and four or five mixtures in each

year (Table 2a;b). There were significant differences among orchards in the number of chemical applications (Table 2a;b) with the maximum number of individual chemical exposures per nest in each year being 11 (Table 2a;b).

Adult exposure to chemicals

Prior to sampling, adult males and females were exposed to slightly more individual chemical applications than the chicks and eggs (Table 3). Adults were exposed to between six and eleven individual chemical applications (Table 3). All adults were exposed to between one and three mixed sprays (Table 3).

Organochlorine pesticides, PCBs, lead, arsenic

Lead and arsenic concentrations in livers were very low and not variable among sites. Lead was present at <0.03 - 0.1 ug/g dry weight among all sites. Arsenic was detected at trace concentrations of 0.20-0.25 ug/g dry wt. in livers from all sites.

Bishop et al (this volume) did not find a substantial gradient of contamination among sites except for pp'DDE. Therefore, the analysis of the correlations between persistent contaminant residues and endocrine responses was confined to pp'DDE. There were no significant correlations among pp'DDE concentrations in eggs and any histological parameter, body or organ mass or hormone concentration.

Histology: testes

Preliminary analysis indicated that there were significant differences in the seminiferous tubules and spermatogonia results among control sites but no differences for other parameters. Therefore, individual site results were pooled among control sites for all but seminiferous tubules and spermatogonia. For those non-pooled parameters, results were compared by sites.

There were no correlations between weather and the dates of sampling of tree swallow chicks for histology of testes. Nested ANOVA and Kruskal-Wallis tests indicated that there were no significant differences for testes mass, area or diameter (Table 4). There were no differences

among sprayed sites or among non-sprayed sites in the organization and frequency of the sertoli cells or the occurrence of heterophils. Analysis of pooled results of sprayed and non-sprayed sites for those parameters found no differences between groups (Table 4). No differences were observed in the morphology of Leydig cells. In every bird, these cells were visible in the interstitium (Table 4).

For spermatogonia, there were significant differences in results among non-sprayed sites ($\chi^2 = 9.8$; p=0.007) but not among sprayed sites. Among non-sprayed sites, two of the three sites had significantly more cases where spermatogonia comprised only 20-50% of the cells on the basement membrane (Control 2 = 75% of cases and Control 3 = 100% of cases) compared to Control 1 where all samples had spermatogonia comprising more than 50% of cells on the basement membrane ($\chi^2 = 3.9$; p=0.05 Control 2; $\chi^2 = 4.1$; p=0.04 Control 3). Individual site comparisons found that two of three non-sprayed sites (Control 2 and 3) revealed no significant differences from each of the sprayed sites. However, there was a significantly lower occurrence of spermatogonia that comprised only 20- 50% of the cells on the basement membrane in Control site 1 samples than in samples from two of three orchards (Orchard 1 = 25% and Orchard 3 = 67%) (Orchard 1: $\chi^2 = 3.9$; p=0.05; Orchard 3: $\chi^2 = 3.9$; p=0.05) (Table 4).

Spray exposure variables were not correlated with testes mass, diameter, area or any histological parameter except the development and organization of sertoli cells on the basement membrane. As the total number of mixtures of pesticides sprayed during chick-rearing increased there was a marginally significant decrease in the probability of sertoli cells forming a contiguous layer above the basement membrane and spermatogonia ($\chi^2 = 5.21$; p=0.02; a 10-fold increase in risk of occurrence with every mixed spray event).

Hormone concentrations and body mass

Two chicks, and one adult male and female were sampled from each nest. Hormone concentrations between sibling chicks of the same sex did not have high coefficients of variation(CV). Mean CV among female chicks ranged from 13 to 18% among testosterone, 17

β- estradiol, and T3 concentrations. For male chicks, mean CV ranged from 12.5 to 28% for the same hormones. Therefore, to avoid pseudoreplication and to account for a small sample size of nests per site, in cases where two chicks of the same sex were sampled from one nest, the mean sex hormone concentration was used in the analysis. Among all sites the means were used for 14 nests comprised of one to three nests per site.

For female chicks, body mass correlated negatively with estradiol (R^2 = 0.44; p= 0.005) and positively with T3 concentrations (R^2 = 0.71; p = 0.005) but did not correlate significantly with testosterone. Nested ANOVA showed no differences among spray treatments or sites for testosterone or body mass (Table 5). ANCOVA adjusted for body mass found no significant differences among spray exposures or treatment groups for estradiol or T3.

Nested ANOVA for male tree swallow chicks revealed that for body mass, concentrations of estradiol, and T3 there were no differences among spray exposure groups or sites within those groups (Table 4). Mean T3 in exposure groups was marginally higher in the sprayed group (1.93 +/- 0.4 ng/ml) compared to the non-sprayed group (1.54 +/- 0.4 ng/ml) (p= 0.06). There were positive correlations between T3 and the number of mixtures sprayed during egg development (R^2 = 0.44; p = 0.005) and T3 and the number of total mixed sprays applied during the period of egg plus chick development (R^2=0.54; p = 0.001) (Fig. 1). Spray exposures were not correlated with any other hormone.

There was a weak negative correlation between collection date of the plasma and testosterone concentrations in male chicks (R^2 = 0.35; p = 0.05). Collection date did not correlate with any other hormone or with body mass. ANCOVA adjusted for collection date showed no differences for testosterone among spray groups and sites. There were no significant correlations between testosterone levels and spray exposures nor was spray exposure significant in multiple regression analysis including collection date as a variable.

In adult birds, there were no significant correlations between any hormone concentration and collection date, body mass or any type of spray exposure. Nested ANOVAs of estradiol,

testosterone, and body mass in adult females did not find any significant differences among spray treatments or sites (Table 5). Similarly, nested ANOVA of testosterone and body mass in adult males found no significant differences among spray treatments or sites.

DISCUSSION

At pesticide application rates and mixtures commonly applied to apple orchards in southern Ontario, thyroid hormone concentrations correlated positively with increasing pesticide exposures in male chicks. There was also a marginally significant trend between decreased sertoli cell organization and mixed pesticide spray exposure. In female chicks, a positive correlation between the thyroid hormone T3 and body mass was revealed but there were no correlations with spray exposure. In contrast, hormone concentrations in adult birds did not show any correlations with spray exposures. These results partially support our prediction that these indicators of endocrine function and sexual development would show differences among sprayed and non-sprayed tree swallow chicks and adults.

The positive correlative associations found between T3 and pesticide exposure are consistent with the responses reported in rats and fish exposed to the same types of pesticides used in these apple orchards. In a freshwater catfish (*Clarias batrachus)*, the carbamate insecticide carbaryl increases T3 concentrations in serum but suppresses thyroxine (T4) levels (Sinha et al, 1991a; 1991b). In another catfish species (*Heteropneustes fossilis*), malathion stimulates extrathyroidal conversion of T4 to T3 (Yadav and Singh 1987). In rats, mancozeb induces thyroid gland activity and mass as a result of increased thyrotrophin stimulation (Ivanova-Chermishanka et al., 1968,1969 in Fishbein, 1976; Przezdziehi et al. in Fishbein, 1976). Although organochlorine pesticide concentrations were low in tree swallow eggs their potential for an effect must be considered since pp'DDT can induce hyperthyroidism in passerines (Jefferies and French 1969). These studies did not evaluate the long-term impact of elevated T3 concentrations but if the higher concentrations of T3 in tree swallows are induced by

an accelerated conversion of T4 to T3, then eventual growth and metabolism of the birds could be affected, especially once parent birds have stopped feeding the chicks. However, there were no indications of effects as measured by differences in body masses of pre-fledging chicks.

Pesticides used in these orchards can also manifest a variety of histological effects on testes. In domestic white leghorn roosters (*Gallus gallus*) some thiocarbamate fungicides reduce testes mass and cause delayed maturation and even degeneration of the seminiferous epithelia where the spermatozoa are formed (Rasul and Howell, 1974). The organophosphorus insecticide parathion induces degeneration of spermatogenic cells, and tubular atrophy of testes in rats (Dikshith et al 1978). Notably, the effect on rat testes was more severe when exposure to parathion was combined with the organochlorine lindane (Dikshith et al 1978). Testicular toxicity and delayed development is also documented when domestic and semi-domestic birds are exposed to OPs (Somkuti et al 1987; Maitra and Sarkar 1991; 1993; 1996; Sarkar and Maitra, 1989; 1990). As cholinesterase (ChE) activity decreased in testes and brain of adult male whitethroated munia (*Lonchura malabarica*) exposed to methyl parathion, the number of degenerated germ cells in the seminiferous tubules of these songbirds increased (Maitra and Sarkar 1996). The potential for an impact of OPs on testes development in songbirds is interesting in light of previous studies in these same orchards that found tree swallow chicks experience marginal cholinesterase inhibition and eastern bluebird (*Sailia sailis*) chicks show significant ChE inhibition after OP spray events (Burgess et al 1998).

Although previous studies have not investigated the implications of such histological abnormalities on reproductive success of the birds, abnormal maturation of the spermatocytes might occur if the sertoli cells were disrupted during sexual maturation. Sertoli cells have a supportive, nutritive role in germinal cell development and it is necessary for the sertoli cells to be in intimate contact with the maturing germinal cells throughout their development until the spermatocytes are released into the lumen of the seminiferous tubules (Lofts and Murton, 1973).

However, some studies suggest effects during early development may not be persistent. Some studies on avian testes exposed to pesticides indicate that histological development returns to normal when exposure is discontinued suggesting that some negative effects may be relatively transient (Rasul and Howell, 1973; Krause et al., 1976).

There were no severe indications of effects on sertoli cells in this study. There were differences in the chi-square analysis (no differences in sertoli cell organization between sprayed and non-sprayed) and the correlational analysis which showed a marginal correlation between sertoli cell organization and spray exposures but this may have been due to differences in the statistical approach. The correlational analysis accounts for number of sprays whereas the chi-square approach compared groups on a simpler criteria of 'sprayed' or 'non-sprayed'. Perhaps the sertoli cells of male tree swallow nestlings were exhibiting the first and most sensitive signs of effects of certain pesticides or number of exposures. While sertoli cell development appeared to be either delayed or unusual in sprayed orchard chicks, other aspects of testes tissue appeared similar to control birds. Dosing rats with the organophosphorus (OP) compound tri-o-cresyl phosphate revealed that sertoli cells are the first to show damage at the light and electron microscopic levels (Somkuti et al., 1986). Meanwhile, the ultrastructure of Leydig cells and basal testosterone levels in the testis and serum are unaltered (Somkuti et al., 1986). In snapping turtles sampled from sites with a gradient of organochlorine exposure *in ovo* and in the diet, differences in sexual morphology of males were found without concurrent differences in circulating testosterone and estradiol concentrations (de Solla et al 1998).

Studies on captive animals and on wildlife found that organochlorine pesticides and some less persistent pesticides can alter sex hormone levels in juvenile animals (Guillette et al, 1994) but this was not found in tree swallows nor has this been reported in other birds. Temperature and other weather conditions are also known to influence sex hormone concentrations in birds (Saint Jalme et al 1996). The negative correlation between collection dates and testosterone in male chicks and the positive correlations between collection date, air

temperature, and spray events on chicks (see Bishop et al., this volume) suggests that weather conditions or other factors including spray exposure may have been important although these cannot be determined from this study.

The finding that sex hormones were not different among birds from different spray treatments does not preclude the possibility that alterations could occur in wild birds exposed to these pesticides. The data does suggest either pesticides have no effect on tree swallow sex hormones, or, exposures to pesticides and/or sampling did not occur at a time when hormone production would be sensitive to an effect. Passerine sex hormones show a transient elevation during which gametogenesis takes place followed by a return to basal levels within a few weeks of clutch completion (Temple, 1974). Tree swallows were not sampled to assess if differences would occur during that critical time. However, in these orchards spray events rarely occurred during the courtship period for tree swallows (Bishop, unpublished) therefore direct exposure during peak sex hormone production may not occur. Nesting adult birds and birds fledged at the study sites often return (Bishop, unpublished). Therefore, an effect could be occurring if exposure the previous year were to affect hormone production at a later time. The study was also biased in that adult birds which were unsuccessful in mating and producing young were not sampled. Effects on testes differentiation in white-throated munia were extremely severe when exposed to methyl parathion during sexual maturation just prior to mating (Maitra and Sarkar 1996). This part of the population might represent the target group, if an effect was occurring. Sampling this portion of the male population might be most intriguing. Also, the order of oviposition within nests was not determined and the relative sibling age among the chicks in each nest may have been important in this study. Since laying order can influence relative sex hormone concentrations among passerine chicks in the same nests (Schwabl 1996; Schwabl et al 1997), this may have elevated the variance in the results.

In conclusion, exposure to pesticides used in these orchards stimulates the production of T3 in male tree swallows. There was some indication that sertoli cell development was

increasingly abnormal as pesticide exposure, and presumably exposure to chicks, increased. These effects on sertoli cells require further study. Immune function in these populations also indicates stimulation of some immunological parameters as well as a delay in thymic involution in tree swallow chicks (Bishop et al this volume). Stimulated immune activity and thyroid hormone concentrations may be induced by suppression of corticosterone which is sensitive to contaminant exposure (Hontela 1997) therefore further study of the avian adenohypophysis axis response to pesticides appears warranted.

The exact pesticide compounds involved in the responses found are difficult to define due to the wide variety of mixtures sprayed from one year to the next in orchards. Further work needs to evaluate the long-term impacts of, at least, the types of mixtures regularly sprayed such as the combinations of dithiocarbamate and ergosterol biosynthesis inhibiting fungicides, and/or the insecticide and fungicide mixtures. The data indicated that decreasing exposures to these common mixtures will reduce the effects in these wild birds. Perhaps through dialogue with farmers and agricultural extension staff and further integrated pest management practices this could be tested although preliminary laboratory studies would be valuable in identifying the exact mixtures and pesticides potentially responsible for these effects.

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Table 1. Pesticides sprayed alone or as mixtures in apple orchards in southern Ontario (1995; 1996) and their application rates

Chemical Activity	Type of Chemical	Trade Name	Active Chemical
Fungicide	EBDC	Polyram	Metiram
Fungicide	EBDC	Dithane DG, M-45	Mancozeb
Fungicide	EBI	Nova 40 W	Myclobutanil
Insecticide	Che Inhibitor	Guthion 50% WP or S	Azinphos-methyl
Insecticide	Che Inhibitor	Imidan	Diazinon
Insecticide / Growth Regulator	Che Inhibitor / Growth Regulator	Sevin 50% WP	Carbaryl
Insecticide	Synthetic Pyrethroid	Decis 5 EC	Deltamethrin
Insecticide	Synthetic Pyrethroid	Ripcord 400 EC	Cypermethrin
Acaricide	tetrazine	Apollo	Clofentezine
Insecticide/ Acaricide	Petroleum Hydrocarbon	Superior Oil	Oil
Herbicide	Herbicide	Round Up	Glyphosate
Growth Regulator	Growth Regulator	NAA	NAA
Nutrient	Nutrient	Zinc (Zn 50)	Zinc (Zn 50)
Nutrient	Nutrient	Boron	Boron
Nutrient	Nutrient	Calcium	Calcium
Nutrient	Nutrient	Nitrogen	Nitrogen
Growth Regulator	Growth Regulator	Sorbatran	1,2-dihydro-3,6-perdazinedion
Growth Regulator	Growth Regulator	Accel	

EBDC = ethylene-bis-dithiocarbamate fungicide
EBI= Ergosterol biosynthesis inhibiting fungicide
NAA = Naphthaleneacetic acid; Che Inhibitor = Cholinesterase Inhibitor

Table 1(CONTINUED). Pesticides sprayed alone or as mixtures in apple orchards in southern Ontario (1995; 1996) and their application rates

Active Chemical	Year Applied	Application Rate per Hectare *
Metiram	95/96	6.0 kg
Mancozeb	95/96	3.0-6.0 kg
Myclobutanil	95/96	340 g
Azinphos-methyl	95/96	2.0 kg
Diazinon	95/96	3.75 kg
Carbaryl	95/96	2.0-3.0 kg
Deltamethrin	95/96	250 ml
Cypermethrin	95/96	250 ml
Clofentezine	95	300 ml
Oil	95	20 L /1000 L tank water
Glyphosate	95/96	0.8 - 1.2 kg
NAA	95/96	5-20 ppm
Zinc (Zn 50)	95	3.6 kg / 1000 L tank water
Boron	95	0.41 L
Calcium	95	5.0 L
Nitrogen	95	5.0 L / 1000 L tank water
1,2-dihydro-3,6-perdazinedione	95	4.5 L
	96	5-20 ppm

* Application Rate per Hectare = based on spray schedules provided by farmers. These rates were used in individual applications and mixtures of chemicals.

Table 1 (CONTINUED). Pesticides sprayed alone or as mixtures in apple orchards in southern Ontario (1995; 1996) and their application rates

Mixtures Applied **	Year Applied
Polyram/Nova	95/96
Dithane/Nova	95/96
Nova/Ripcord	95/96
Dithane/Ripcord/Boron	95
Dithane/Boron	95
Zinc/Polyram	95
Nova/Dithane/Foliar Nitrogen	95
Dithane/Sevin	95
Dithane/Apollo/Sniper/Sevin/Calcium	95
Dithane/Deltamethrin	96
Nova/Ripcord	96
Imidan/Myclobutanil	96
Nova/Dithane/Sniper	96
Guthion/Polyram	96
Imidan/Polyram	96
Dithane/Imidan	96

** Mixtures Applied = chemical applied as a tank mixture or separate sprays but applied on the same date.

There are no recommended OMAFR rates for the applications of mixtures. For each chemical, farmers apply the same rates in mixed sprays as applied in a single chemical spray event.

Table 2a. Mean (Standard Deviation), maximum and minimum number of individual chemical exposures per nest during egg and chick stages of tree swallows sampled for hormone analysis or testes evaluation in four apple orchards in southern Ontario (1995; 1996)

		No. Individual Chemical Applications :				Sum total of Individual Chemical applications during Egg Stage & Chick-rearing		N	N
		Egg development	Egg development	Chick-rearing	Chick-rearing				
		1995	1996	1995	1996	1995	1996	1995	1996
Orchard									
1	**Mean (St. Dev)**	4.9 (1.1) A	2.5 (1.0)A	3.2 (1.3) A	3.5 (0.5)A	8.0 (1.5) B	6.2 (0.5)B	7	4
	min.; max.	4 ; 7	2 ; 4	2 ; 6	3 ; 4	7 ; 11	6 ; 7		
2	**Mean (St. Dev)**	4.5 (1.8) A	5.8 (1.2)B	3.8 (0.4) A	4.8 (1.2)A	8.3 (1.8) B	10.6 (0.8)A	6	6
	min.; max.	1 ; 6	4 ; 7	3 ; 4	4 ; 7	5 ;10	9 ; 11		
3	**Mean (St. Dev)**	2.2 (1.5) B	1.8 (0.4)A	3.4 (0.5) A	3.2 (0.4)B	5.7 (1.1) A	5.0 (0)C	9	6
	min.; max.	1 ; 4	1 ; 2	3 ; 4	3 ; 4	4 ; 7	5 ; 5		

N = number of nests sampled

Different letters (A, B etc.) among sites within year and parameter indicate significant differences $p <= 0.05$

Table 2b. Mean (Standard Deviation), maximum and minimum number of mixed chemical exposures per nest during egg and chick stages of tree swallows sampled for hormone analysis or testes evaluation in four apple orchards in southern Ontario (1995; 1996)

		No. Mixed Spray Events:				Sum total of Mixed Sprays			
		Egg development	Egg development	Chick-rearing	Chick-rearing	during Egg Stage & Chick-rearing		N	N
		1995 *	1996	1995 *	1996 *	1995	1996	1995	1996
Orchard									
1	**Mean (St. Dev)**	2.6 (0.5)	1.0 (0)B	1.0 (0.6)	1.0 (0) A	2.6 (0.5) A	2.0 (0) B	7	4
	min.; max.	2 ; 3	1 ; 1	0 ; 2	1 ; 1	2 ; 3	2 ; 2		
2	**Mean (St. Dev)**	1.0 (0)	3.2 (0.8)A	0.7 (0.5)	1.6 (0.8) A	1.0 (0) A	4.8 (0.4) A	6	6
	min.; max.	1 ; 1	2 ; 4	0 ; 1	2 ; 4	1 ; 1	4 ; 5		
3	**Mean (St. Dev)**	1.1 (0.3)	0.2 (0.4)B	0.7 (0.7)	0 B	1.1 (0.3) A	0.2 (0.4) C	9	6
	min.; max.	1 ; 2	0 ; 1	0 ; 2	0 ; 0	1 ; 2	0 ; 1		

N = number of nests sampled

* = statistical analysis not performed to compare among sites becasue there were too few spray events per site

Different letters (A, B etc.) among sites within year and parameter indicate significant differences $p <= 0.05$

Table 3. Mean (Standard Deviation), maximum and minimum number of chemical exposures to adult male and female tree swallows sampled for hormone analysis in four apple orchards in southern Ontario (1995)

		No. Individual Chemical Applications		No. Mixed Spray Events:			
		Males	Females	Males	Females	N Females	N Males
		1995	1995	1995	1995		
Orchard							
1	**Mean (St. Dev)**	9.6 (1.1)A	8.6 (1.4) A	3 (0)A	2.5 (0.5)A	6	3
	min.; max.	9 ; 11	7 ; 11	3; 3	2 ; 3		
2	**Mean (St. Dev)**	6.5 (0.6)B	6.5 (0.5)B	2 (0)B	2 (0)B	7	4
	min.; max.	6 ; 7	6 ; 7	2 ; 2	2 ; 2		
3	**Mean (St. Dev)**	9 (0)A	6.6 (0.5)B	2.8 (0.5)A	1 (0)C	7	4
	min.; max.	9 ; 9	6 ; 7	2 ; 3	1 ; 1		
4	**Mean (St. Dev)**	NS	6.8 (0.4)B	NS	1 (0)C	6	NS
	min.; max.		6 ; 7		1 ; 1		

NS = not sampled in that year
N = number of adults sampled
Different letters (A, B etc.) among sites within year and parameter indicate significant differences $p <= 0.05$

Table 4. Histological assessment and testes mass and size in male tree swallow chicks from nests in sprayed orchards and non-sprayed sites in southern Ontario, 1996.

	SPRAYED ORCHARDS N = 16 nests *		NON-SPRAYED SITES N = 14 nests*	
Testes Mass and Size				
Mass (mg) **	2.06 (0.63)		2.29 (0.6)	
Diameter (mm)	0.71 (0.20)		0.68 (0.19)	
Area (sq.mm)	0.68 (0.27)		0.63 (0.19)	
Histopathology				
No. seminiferous tubules in largest testis per chick	57.2 (21.8)		45.0 (20.2)	
Sertoli Cells				
a) Cells form a discontinuous layer above basement membrane	25.0 %		15.4%	
b) Cells form a contiguous layer above the basement membrane	75.0%		84.6%	
Leydig Cells visible in interstitium	100%		100%	
Spermatogonia				
a) comprise 20-50% of cells on basement membrane	62.5 %	***	38.46%	***
b) comprising greater than 50% of cells on basement membrane	37.5%	***	61.54%	***
Presence of Heterophils				
a) 0-2 heterophils	57.14%		31.25%	
b) 3-6 heterophils	35.71%		56.25%	
c) focus of heterophils present in interstitium	7.14%		12.5%	

* 1 chick sampled per nest

** Sample size for weights were N=11 for non-sprayed sites; N=15 for sprayed orchards

*** Significant differences did not exist among orchard and among non-sprayed sites for any parameter except spermatogonia. For spermatogonia, Control 1 and Orchard 2 were not significantly different but Control 1 had significantly higher occurrence of spermatogonia comprising >50% of the cells on the basement membrane than all other sites and Orchard 1,2,3 and Control 2,3 were not significantly different ($p<=0.05$).

Table 5. Body mass, sex and thyroid hormone concentrations (Mean (Standard Deviation)) in tree swallows from sprayed orchards and non-sprayed sites, Southern Ontario, Canada (1995).

		BODY MA		TESTOSTERONE (pg/ml)	
		SPRAYED ORCHARD	NON-SPRAYED SITE	SPRAYED ORCHARD	NON-SPRAYED SITE
16 - DAY OLD CHICKS:					
FEMALE	Mean	20.2	20.7	530	566.8
	St.Dev.	(1.3)	(1.4)	(200.5)	(287.1)
	N	15	17	7	9
MALE	Mean	21.9	20.7	490.5	514.7
	St.Dev.	(1.3)	(1.4)	(181.4)	(92.9)
	N	13	17	11	7
ADULTS:					
FEMALE	Mean	22.9	23.4	424.9	862.2
	St.Dev.	(2.4)	(1.5)	(292.5)	(1234.0)
	N	32	18	26	18
MALE	Mean	21.0	20.4	2052.1	2551.2
	St.Dev.	(1.2)	(1.0)	(987.1)	(1665.6)
	N	13	13	11	10

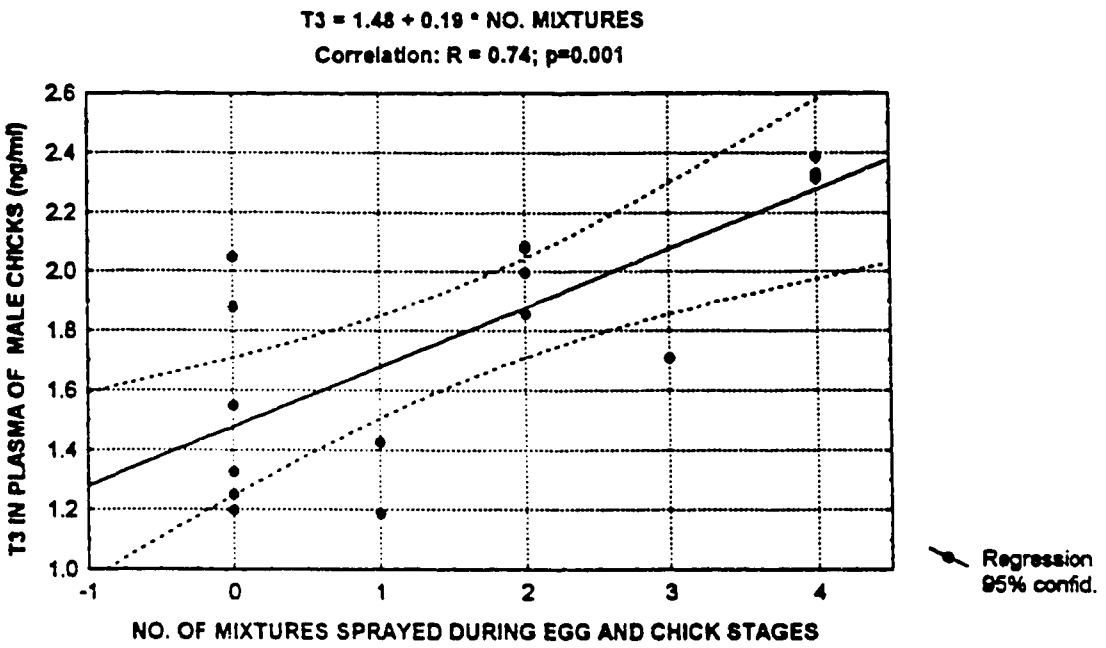
NM = Not Measured; N/A = Not Applicable; N = Number of Nests Sampled
Note: Between orchard and non-sprayed sites, we found no significant differences for each parameter within age groups and sexes ($p <= 0.05$)

Table 5 (CONTINUED). Body mass, sex and thyroid hormone concentrations (Mean (Standard Deviation)) in tree swallows from sprayed orchards and non-sprayed sites, Southern Ontario, Canada (1995).

		17 B- ESTRADIOL (pg/ml)		TRIIODO-L-THYRONINE T3 (ng/ml)	
		SPRAYED ORCHARD	NON-SPRAYED SITE	SPRAYED ORCHARD	NON-SPRAYED SITE
16 - DAY OLD CHICKS:					
FEMALE	Mean	1523.4	1591.1	1.96	1.56
	St.Dev.	(786.9)	(357.4)	(0.6)	(0.4)
	N	7	9	4	8
MALE	Mean	1575.8	1250.8	1.93	1.54
	St.Dev.	(517.1)	(205.1)	(0.4)	(0.4)
	N	11	5	10	6
ADULTS:					
FEMALE	Mean	1695.5	1572.5	NM	NM
	St.Dev.	(558.2)	(416.7)		
	N	26	18		
MALE	Mean	NM	NM	NM	NM
	St.Dev.				
	N				

NM = Not Measured; N/A = Not Applicable; N = Number of Nests Sampled
Note: Between orchard and non-sprayed sites, we found no significant differences for each parameter within age groups and sexes ($p \leq 0.05$)

Figure 1. Spray mixtures applied during egg and chick stages vs. T3 in male chicks.


T3 = 1.48 + 0.19 * NO. MIXTURES
Correlation: R = 0.74; p=0.001
T3 IN PLASMA OF MALE CHICKS (ng/ml)
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
-1
0
1
2
3
4
NO. OF MIXTURES SPRAYED DURING EGG AND CHICK STAGES
Regression
95% confid.

Chapter Three.

The effects of pesticide spraying on chick growth, behaviour and parental care in tree swallows (*Tachycineta bicolor*) nesting in apple orchards in Ontario, Canada

The Effects of Pesticide Spraying on Chick Growth, Behaviour and Parental Care in Tree Swallows (*Tachycineta Bicolor*) Nesting in Apple Orchards in Ontario, Canada

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SUMMARY

(1)Our objective was to investigate the consequences of pesticide use in an apple orchard on tree swallow (*Tachycineta bicolor*) behaviour and growth.

(2) In a sprayed apple orchard and two non-sprayed sites in southern Ontario, Canada in 1996-1997, hunger signalling and growth of chicks, feeding and incubation activities of adults, and aerial invertebrate abundance were studied before and after pesticide spray events.

(3) The insecticides carbaryl and azinphosmethyl ($\leq 0.091 ug/cm^2$) deposited on filter papers placed in nest boxes during spray events.

(4) There were no significant differences among sites in adult incubation times. However, in the orchard there were persistent and significant increases in hunger signalling by tree swallow chicks after organophosphorus insecticide (OP) spray events in both years and, after a second OP spray in 1996, significant decreases in the number of feeding trips by parent birds. These trends did not occur in the control sites.

There were few differences among sites in the size and type of aerial invertebrates caught in traps. There were very low densities of invertebrates in the orchard relative to the other locations but the abundance did not vary significantly with insecticide events.

(5) After OP spray events in the orchard, the trends in feeding sorties of adult birds during chick-rearing did not coincide with known experimental responses in tree swallows subjected to reduced food resources. These trends were consistent with those predicted to occur in passerines exposed to cholinesterase-inhibiting insecticides. Conversely, the increased hunger signalling may reflect chronically low food resources available in the orchard but not cholinesterase-depression.

Despite these changes in behaviours, differences in masses of chicks among sites and after spray events were not related to pesticide use in the orchard. Tree swallows may be able to feed chicks adequately because there were local, non-sprayed habitats available for feeding. Since first year survival of tree swallow chicks is correlated with their mass at fledging this suggests a need to maintain relatively natural habitats near sprayed agricultural areas.

INTRODUCTION

Organophosphorus (OP) and carbamate (CARB) insecticide exposure can depress avian cholinesterase (ChE) levels which can sometimes lead to the death of wild birds in agricultural areas including orchards (Wilson et al 1997; Hooper et al. 1989). The sublethal effects of these chemicals on birds includes reduced egg production, hatchability of eggs and fledging of young in captive and wild birds (Smith 1987; Patnode and White 1991; Fleutsch and Sparling 1994) yet the mechanism(s) of altered reproductive success remain unclear.

Evidence shows that sublethal exposure to cholinesterase-inhibiting insecticides can change bird behaviour and it has been proposed that this could affect their survival or parenting abilities (Grue, Powell & McChesney 1982; Grue, Gilbert & Seeley 1997). In wild European starlings *(Sturnus vulgaris)* sublethally dosed with the OP insecticide dicrotophos, feeding trips and chick mass gains were significantly reduced within 12-24 hours after exposure (Grue, Powell & McChesney 1982). In the first few hours after oral exposure to chlorfenvinphos, Hart (1993) found altered posture, increased resting, decreased flying and an accompanying loss of body mass in captive starlings. Similarly, reduced incubation time was found in laughing gulls (*Larus atricilla*) (White, Mitchell & Hill 1983) and red-winged blackbirds *(Agelaius phoeniceus)* (Meyers, Cummings & Bennett 1990) dosed with organophosphorus insecticides but the effects persisted for only a few hours.

Studies also show that arthropod and other invertebrate food sources for birds are reduced after insecticide spray events (Powell 1984). Reductions in food due to pollution can affect reproductive success in some species of birds (Eeva, Lehikonen & Pohjalainen 1997) and not others (Rodenhouse & Holmes 1992; Eeva, Lehikonen & Pohjalainen 1997) whereas the direct effects of the contaminants can be a more important factor in reproductive success in some species (Eeva, Lehikonen & Pohjalainen 1997). For these reasons, it has been suggested that the direct effects of cholinesterase-inhibiting insecticides on parent and nestling birds may be exacerbated by indirect effects on their behaviour and/or food resources on or near farms

(Grue, Powell & McChesney 1982).Since depressed cholinesterase levels (Burgess *et al.* In press) and lower egg viability (Bishop *et al.*, submitted) are found in orchard-nesting tree swallows (*Tachycineta bicolor*), our study monitored hunger signalling and growth of tree swallow chicks as well as feeding activities and incubation periods of adult swallows in a pesticide-sprayed apple orchard. Tree swallows are sensitive to arthropod density (Hussell 1988) therefore aerial invertebrate abundance at these sites was also measured. It was predicted that there would be significant differences in all of these parameters at the orchard compared to the non-sprayed sites.

STUDY AREAS

One apple orchard site and two non-sprayed, non-orchard sites (Control 1 and 2) were studied during May- late June in 1996 and 1997. These sites were located within a 10-20 km radius from St. George, Ontario, Canada (approximately 43°15'/ 80°2'). Both non-sprayed sites were cattle pastures. The cattle maintain the vegetation at a low height (0.1-0.5 m) which is similar to the height of the herbaceous vegetation within the orchard. The orchard was mowed between tree rows and herbicide was applied once a month to vegetation between the trees. All sites had ponds (0.5-1.0 ha) within 250m of the nest site.

Nest boxes were established and occupied for at least one year prior to the first year of our study. Each nest box was 8-10m from its nearest neighbour and entrances to all nest boxes faced south to avoid the prevailing wind. Nest predators were deterred by applying axle grease to nest box poles prior to the start of breeding season. The nest boxes in the orchards were located between apple trees.

METHODS

Nesting Phenology, Selection of Study Nests, Handling of Birds

During the egg-laying period, breeding activity was monitored at each nest on a daily basis. In an attempt to remove any effect of weather conditions among observation days and nests (Lombardo *et al.* 1995; McCarty 1995), avian and invertebrate data were collected within the same period (+/- 1 hour) at all sites. In each year, nests were selected for study based on

their synchrony of reproductive cycle among sites during incubation and chick-rearing. In 1996, all nests had first eggs during 19-21 May and hatched during 6-9 June (Appendix 1: Table 1). In 1997, first eggs were laid during 20-26 May and hatched 8-14 June in 1997 (Appendix 1: Table 1). Five or six nests were selected per site for observation in each year (Appendix 1: Table 1) however, during the study in 1997, two nests at Control 2 were lost to predation during the study.

Because feathers that line tree swallow nests have significant insulative capacities and can influence incubation period and chick growth (Lombardo *et al* 1995), the number of feathers in each nest (+/- 5 feathers) was counted during the second to fourth days of incubation in each nest. Using the Kruskall-Wallis test (Sokal & Rohlf 1981), there were no significant differences found in the number of feathers in nests among all three study sites in each year (Appendix 1: Table 1).

Based on plumage characteristics, breeding female tree swallows were classified as second year (SY, mostly brown dorsal plumage), after second year (ASY, mostly iridescent blue-green dorsal plumage), or after hatching year (AHY ie. intermediate plumage; Hussell 1983). Males develop full adult breeding plumage before their first winter (Robertson, Stutchbury & Cohen 1992). The age of females was noted because second year females can have lower reproductive success compared to after second year females (Hussell 1988). Nests were attended by both ASY and SY birds at all sites although the orchard site had fewer SY birds than one of the control sites in 1996 (Appendix 1: Table 1). All birds were handled according to the Canadian Council on Animal Care (Olfert, Cross & McWilliams 1993) and procedures were approved by the Canadian Wildlife Service Animal Care Committee.

Statistical Approach

Data were log-transformed, where necessary, to meet normality and homogeneity of variance requirements for parametric analysis. If those criteria could not be met, non-parametric tests were used. All data were analyzed using STATISTICA for Windows (Statsoft, 1997). Results were considered statistically significant at $p=0.05$, however, to avoid type I errors in many correlations performed on results of feeding trips, calling time and time adult birds spent

in nest boxes, the significance of these results were determined using a sequential Bonferroni test on p=0.05 for four variables (Sokal & Rohlf 1981; Rice 1989).

Spray Event and Observation Phenology

Pesticides in the orchard were applied with an air-blast sprayer by the orchard owner who is a licenced pesticide applicator. The chemicals were applied according to the farmer's choice of spray schedule which depended on pest cycles in the orchard and advisories from the Ontario Ministry of Agriculture and Rural Affairs (OMAFR) 'agri-phone' and Fruit Production Recommendations (OMAFR, 1996). Therefore, the study site was generally representative of chemical application methods and schedules used in apple orchards in Ontario. The same orchard was studied in both years.

Observations were made during the daylight hours several days before and, if possible, several hours immediately prior to the spray event (see pre-spray periods Table 1). Since the fungicides used in this study have low acute toxicity and are not cholinesterase-inhibiting (Fishbein, 1976) nor do they directly affect insects, monitoring incubation after these spray events in the orchard and after water-spray events in the control sites allowed us to test the effect of a disturbance due to a spray event. Because sublethal effects can occur within hours in songbirds dosed with cholinesterase-inhibiting insecticides (Grue, Powell & McChesney 1982; Meyers, Cummings & Bennett 1990; Hart 1993), the nests were observed within 1.5 to 6 hours after spray events (Table 1). Insecticides used in this study were expected to remain toxic for at least 48 hours of application (OMAFR, 1996). Therefore the post-spray periods were considered a period of at least 48 hours after spray events.

Incubation period

Neither egg manipulations nor the presence of foreign, conspecific eggs affect duration of incubation period in tree swallows (Burtt 1977). However, clutch size can have an effect on incubation time in some passerines (Biebach 1984), therefore clutch sizes were standardized in all nests to five eggs in 1996 (Appendix 1: Table 1). Two days after the clutch was completed, this involved removing one egg from some nests (Appendix 1: Table 1).

In tree swallows, incubation starts on the day the last egg is laid and hatching occurs 14 days later (Robertson, Stutchbury & Cohen 1992). The total incubation period (+/- 12 hours) was determined for each nest and the length of these periods was compared among sites using the Kruskal-Wallis test (Sokal & Rohlf 1981). Binoculars were used to observe nests for 30 minute periods with the same observer at each site throughout the study. The time spent incubating by females was determined by timing with a digital watch (+/- 0.5 s) the period females spent in the nest box. The presence of a male bird was noted and confirmed in the vicinity of each nest. It was also noted that other female birds did not enter and remain in the nest box during observation periods.

Chick-rearing period

Individual songbirds within large broods may show reduced survival or body mass (Hussell 1972; Askenmo 1977). However, broods raised by foster parent tree swallows grow just as well as those raised by natural parents (Quinney, Hussell & Ankney 1986). Therefore, when nestlings were five days old in 1996, brood sizes were adjusted to five chicks per nest, if necessary (Appendix 1: Table 1). In 1997, brood size was adjusted to six chicks per nest within 24 hours of hatching (Appendix 1: Table 1). To increase brood size, chicks of equal age from the same site were added to the nest.

Observations of Nests: Feeding Trips

Since videotapes of tree swallow nest boxes show that 100% of trips made into boxes during chick-rearing involve food delivery to twelve day-old chicks (McCarty 1995), the nests were observed for 30 min periods and the total number of feeding trips was counted and the time spent in the box by male and female parents was measured. The same observer conducted observations at only one site throughout the season. Each trip into a nest box was assumed to represent a feeding trip unless an unusual male or female bird was noted at the nest. This behaviour was seen on less than four occasions and the data for those occasions were not used in our analysis. If the behaviour occurred more than once at a nest, the data for that nest were not used in our analysis. Observations of nests were conducted at the same distances from the

boxes as the incubation study.

Calling Time by Chicks and Feeding Trips monitored by Tape Recordings

Since time spent calling by nestling birds is indicative of hunger signalling (Hussell 1988; Godfray 1991), a battery-operated tape recorder (Sanyo microcassette M-5699C; 12 x 4 x 2 cm) was installed directly above each nest to measure the calling time of the brood. Recorders were placed in the boxes on, at least, a daily basis after chicks were four days old (after 17 June 96 and after 9 June 97). Thirty minute tapes were used when chicks were five to six days old in 1996. When chicks were older than six days in 1996 and during all monitoring in 1997, tapes recorded for 60 to 62 minutes. When chicks were older than four days, the entrance of a parent bird into the nest box and even a single chick call were audible on tape and easily differentiated from parental calls (Robertson, Stutchbury & Cohen 1992). Based on the tapes, the number of feeding trips was counted and the calling time for chicks was timed. To account for the variation among boxes in the time required for the parents and/or chicks to recover from the disturbance of the installation of the tape recorder, the number of feeding trips per second was based on the total time the tape ran after the first time a parent bird entered the nest box. The calling time per second (+/- 0.5s) was based on the total time the tape ran after a) the first time a chick called and b) the first time a parent bird entered the nest box. Usually parents entered the box within ten to fifteen minutes after our disturbance.

Body mass

The mass of each chick was measured with a 50 (+/- 0.5) g Pesola scale, at least, every 24 hours. Masses of chicks were highly correlated with their age at all sites and in both years (Spearman Rank $R=0.81$ to 0.86 among sites; $p \leq 0.05$). Therefore, using multiple regression to account for chick age (Sokal & Rohlf 1981), differences among sites in each year in mean, log-transformed masses of broods were determined.

Differences among sites in the percentage change in mean masses of broods after insecticide sprays were applied was also determined. The percentage change in mass for 24 and 48 hours after each spray event relative to the mass measured in the observation period

immediately prior to the spray event was calculated (Table 2). Results were compared among sites by the Kruskal-Wallis test (Sokal & Rohlf 1981).

In 1996, chick masses were measured on and after 14 June when chicks were five days old until 48 hours after the last azinphosmethyl spray when chicks were 13-14 days of age. In 1997, chicks were weighed at 24 hours of age and every 24 hours thereafter until one week after the insecticide spray event.

Aerial Invertebrate Abundance

Aerial invertebrates were trapped in suspended, stationary, aerial tow-nets which were the same as those used by Quinney & Ankney (1985). The invertebrate catch in this type of funnel trap correlates with the proportion of invertebrate taxa delivered to tree swallow chicks (Quinney & Ankney 1985). Two nets were placed at approximately 1.8-2 m above ground, 50m apart, and among the nest boxes in each study site.

Invertebrate density was compared among sites based on the number of invertebrates caught in each trap per km of wind that passed through the trap. Wind measurements in kilometres per hour were taken at each site on three occasions using hand held anemometers. Regression equations were then determined to calculate the total kilometres per hour of wind that went through each trap per day based on hourly wind speeds recorded at the local weather station at the Royal Botanical Gardens, Burlington, Ontario compared to wind measurements taken at the study sites ($R=0.80$; $p=0.04$)(Quinney & Ankney 1985).

All invertebrates in each sample were identified to order and Diptera were identified to suborder Nematocera or other suborders (according to Brues & Melander 1915). Two orders of the Class Arachnida were also identified Araneae (spiders) and Acarina (mites and ticks).

In each site in 1996, invertebrates were sampled during 0700 hrs to 1530 hrs each day for a maximum of 32 days during 13 May to 21 June. In each site in 1997, invertebrates were sampled during 0700 hrs to 1530 hrs each day for a maximum of 27 days during 19 May to 23 June at each site.

Pesticide residues

To determine if pesticides entered the nest box and deposited in the nest area, filter papers (90 mm diam; Type A/E; Lot. 2358; Gelman Sciences, Ann Arbor, Mn) were placed in nest boxes at all sites during all azinphosmethyl spray events in both years and during the carbaryl event in 1997. All measurements were performed on generically coded samples to facilitate blind analyses. One filter paper per nest-box was sampled. Filter papers were placed in glass petri dishes previously cleaned with non-phosphorus soap, hexane and petroleum ether. The petri dish and contents were placed on wooden blocks approximately 8 cm high so that the filter papers would simulate the approximate height and location of the nest cup. Samples were collected in nest boxes distributed throughout the orchard. For all but one spray event filter papers were collected within 30 minutes after the spray event. The exception among spray events was samples exposed at 2130 hrs and left in the nest boxes overnight and collected 10 hours after the Carbaryl spray event in 1997. Once collected from the nest box with tweezers previously cleaned with non-phosphorus soap, hexane and petroleum ether, filter paper and petri dishes were wrapped in chemically-cleaned foil and samples were immediately placed on ice packs in a cooler and either taken directly to the analytical laboratory or frozen overnight at -5°C and delivered to the laboratory the next day.

To analyze for carbaryl, filter papers were soaked overnight in dichloromethane followed by filtration and concentration. Carbaryl was determined using a high pressure liquid chromatograph with post-column derivatization and flourescence detection (United States Food and Drug Administration (USFDA), 1994). Recovery of internal carbaryl standards was 80-100%. The detection limit for carbaryl was 0.2 ug.

To analyze for azinphosmethyl, filter papers were soaked overnight in dichloromethane followed by filtration and concentration. Extracts were cleaned and fractionated on florisil as described by Mills et al. (1972). Azinphosmethyl was determined using a GC coupled to a flame photometric detector in the phosphorus mode (USFDA, 1994). The detection limit for the analysis was 0.2 ug. Recoveries of internal azinphos methyl standards were 85-100%.

For organochlorine residues, previous sampling had indicated that the concentrations of organochlorine pesticides in eggs of tree swallows at our study sites were low (< 0.6 ug/g wet weight) (Bishop *et al.* unpublished).

RESULTS

In 1996, there were no significant differences among sites in the total number of days (+/- 12 hr) from beginning of incubation to hatching in nests among sites (Table 2). Complete hatching occurred in all clutches. During incubation in 1997, there were two spray events. Cypermethrin, mancozeb and myclobutanil were sprayed followed by a mancozeb spray (Table 2). Avian incubation periods were monitored in 1997, but, nest initiation was extremely asynchronous among sites, therefore nests would not be adequately comparable. In both 1996 and 1997, all chicks survived to the end of the study.

During the 12 days when observations were made during the incubation period there was a positive, significant Spearman rank correlation between incubation day and incubation time per 30 minute period at Control 2 ($R=0.62$; $p=.002$) but not any other site. There were no correlations at any site between incubation time and day of incubation period for the three days before the spray events to four days afterward. Therefore, the incubation times were compared among sites and the percentage change in incubation times after spray events relative to the pre-spray periods using the Kruskal-Wallis test (Sokal & Rohlf 1981).

Incubation Time and Response to Fungicides

In 1996, there was a total of three spray events in which five types of chemicals were applied to the orchard (Table 1). The first spray event was a combination of two fungicides and tree swallow nests were not completed and incubation had not been initiated therefore monitoring of swallow behaviour was not performed (Table 1). The second spray event was the application of an ethylene-bis-dithiocarbamate (EBDC) fungicide (Table 1). On the same day and time, water was sprayed with a hand held sprayer for one minute on each nest box at both control sites. Nests were at the fifth to seventh day of incubation.

Mean incubation time prior to the spray event ranged from 20.6 to 21.5 min./ 30 min

observation period among sites (Table 2). After the spray, mean incubation time at the orchard ranged from 12.7 to 23.6 min. At the control sites, mean observation time ranged from 13.8 to 23.4 min (Table 2). Among sites, there were no significant differences in incubation times during each observation period. There was only one period in which there were significant differences among sites in the percentage change in incubation time after the spray event relative to the pre-spray period. Forty-eight hours after the spray event, the percentage change in incubation time at Control 2 was significantly lower than at the Orchard or Control 1. These results indicated that there was no effect of either a fungicide spray event or water sprayed on nest boxes on incubation activity.

Incubation Time and Response to Insecticides

The last spray event (Table 1) during incubation was the application of azinphosmethyl, an OP insectide. The event occurred within 24 hours of the hatching date of some nests in the study therefore our sample size was reduced at each site (Table 2). After the spray event, mean incubation time in the orchard ranged from 17.3 to 26.8 min. At the control sites, mean observation time ranged from 13.0 to 25.3 min (Table 2). At 1.5 hours after the spray event, Control 2 had incubation times that were significantly lower compared to the other two sites. The mean of the percentage change in incubation at 24 hours after the spray event was significantly lower at Control 2 compared to Control 1, but not different than the Orchard. At 29 hours after the spray event the percentage change at Control 1 was significantly higher than Control 2 but not the orchard.

Feeding Trips and Time Spent in Nest-boxes by Adults and Chick Calling Time And Response to Insecticides

When the entire chick-rearing period was considered in each year, there were significant Spearman rank correlations between chick age at the control sites and the number of feeding trips, time spent by the parents in the nest box, and calling time of the chicks (Table 3a,b). Therefore, it was determined whether such trends with chick age were consistent at the orchard site during a) the entire chick-rearing period during our study and b) specific periods before and

after spray events in each year (Table 3a,b). It was assumed that significant trends that were consistent between the control sites but different from the sprayed site may be associated with an effect of the chemical. Significance of correlations was tested with Spearman Rank values for individual nests and the Approximate Z-test with a sequential Bonferroni test of p=0.05 for four variables (Rice 1989; Sokal & Rohlf 1981). However, since nests were well matched for age of the chicks an additional analysis was also performed to examine effects within 24 hours after the spray event. A Kruskal-Wallis test was used to perform a comparison among sites in the percentage change in the number of feeding trips / 30 min observation period in the post-spray period relative to the pre-spray periods.

During chick-rearing in 1996, seven different chemicals were applied during four spray events (Table 1). The first two spray events were a herbicide and a mixture of two fungicides (Table 1). The chemicals were applied when our study nests were hatching or just a few days later. Monitoring of feeding trips was not initiated until after the fungicide event on 10 June when chicks were approximately one to three days-old. The third spray event was a carbamate insecticide, carbaryl. The last spray was azinphosmethyl (Table 1).

During the chick-rearing period, 30 min field observations showed there was a significant and positive increase in the number of feeding trips/second, relative to chick age, at both control sites for the entire period. The number of feeding trips did not increase as chicks grew older at the orchard site during that time (Table 3a). Similarly, the number of feeding trips/ second tape recorded during the period 17-22 June was significant and positive at Control 1 and marginally (p=0.02-0.05) positive at Control 2. There was no significant trend at the orchard site (Table 3a). Thirty minute observations also showed a decrease at all sites in the time birds remained in the nest box as chick age increased (Table 3a). Conversely, there was a significant increase in calling time of chicks at all sites (Table 3a).

Prior to the carbaryl spray during chick-rearing in 1996, data was collected by observations but not by tape recorder because chicks were too young to be detected on tape. Over the four days prior to the spray the mean number of feeding trips in the orchard was 6.1

feeding trips / 30 min while it was 3.3 feeding trips/ 30 min at Control 1 and 6.6 feeding trips/ 30 min at Control 2. For correlation analysis, data were only collected for two nests for more than two of the four days prior to the spray at the orchard but there was a general decrease in feeding trips over time whereas there was no significant trend at Control 1 and data were not collected for that period at Control 2 (Table 3a).

During the five days after the carbaryl spray event, there was a significant and positive trend in feeding trips per 30 min at Control 1 but not Control 2 or the orchard (Table 3a). Tape recordings showed no significant trend in feeding trips at any site (Table 3a) while 30 min. observations indicated a significant decrease in the time birds remained in the nest box (Table 3a). There was also a significant increase in the calling time at the orchard and Control 2 (Table 3a). Among sites, there was no significant difference among sites in the percentage change in the mean number of feeding trips / 30 min during the 24 hour period after the spray event. At the orchard the mean number of feeding trips / 30 min was 9.9, at Control 1 was 3.6 and at Control 2 it was 5.8.

After the azinphosmethyl spray in 1996 during chick-rearing, there were no significant trends in time spent in the nest box at any site. At the orchard, there was a significant decrease in the number of feeding trips measured by tape recordings and by 30 minute observations. There were no significant trends in feeding trips at either control site although there was a positive trend at Control 2 (Table 3a). The calling time increased significantly at the orchard after the spray while it did not change significantly at the control sites (Table 3a). The mean number of feeding trips / 30 min at the orchard was 8.8 during the pre-spray period and increased to 13.5 during 24 hours after the spray. The mean was 5.7 feeding trips/ 30 min at Control 1 before the spray event and was 6.1 afterward and the mean was 6.6 before spray at Control 2 and 7.5 afterward. However, the percentage change in number of feeding trips after the spray relative to the pre-spray period was not significantly different among sites.

During chick-rearing in 1997, two insecticides were sprayed sequentially on the same day (Spray 3 & 4: Table 1). Azinphosmethyl was sprayed at 1430 hrs and Carbaryl was sprayed

at 2130 hrs on 15 June (Table 1). At the control sites, nests were sprayed with water via a hand-held sprayer at the same time that the orchard was sprayed. In 1997, feeding trips, time spent in the nest box and calling time increased with increasing chick age at all sites (Table 3b). In 1997 during 9-23 June, trends in calling time, and time spent in the nest box were similar among sites. Feeding trips increased over time at all sites although there were some differences among sites in the strength of the correlation between feeding trips and age.

Prior to the day of the insecticide spray events , there were no significant trends found in feeding trips taken or time spent in the nest box relative to chick age (Table 3b). Because chicks were less than five days old in most nests, there were not enough taped measures of feeding trips relative to age for statistical analysis of the pre-spray period.

During three days immediately after the spray, the orchard and Control 1 had a significant positive trend in chick calling (Table 3b).The time spent in the nest boxes declined significantly at Control 1 and the orchard after the spray event. There was still a significant and positive trend in calling time at the orchard whereas this did not occur at the control sites (Table 3b).

Mass of Chicks

During the entire chick-rearing period in 1996, masses of chicks showed significant differences among sites. Control 2 and the orchard had significantly higher age-adjusted masses than Control 1 ($F^{[3,101]}= 108.6$; $R^2=0.74$; $p=0.0001$; Fig. 1a). However, 24 and 48 hours after the carbaryl spray event, there were no significant differences among sites for percentage of mass gain by chicks. Also, there were no marked differences in masses between adjusted and non-adjusted broods for those periods (Table 4). Twenty-four and 48 hours after the azinphosmethyl spray event there were no significant differences among sites in percentage of mass gain by chicks and little difference between adjusted and non-adjusted broods (Table 4).

For the entire chick-rearing period in 1997, age-adjusted masses in Control 2 birds were significantly higher than those in Control 1 and the orchard ($F^{[3,154]}= 709.2$; $R^2=0.93$; $p=0.0001$; Fig. 1b). AS in 1996, there was little variation among mean masses among nests within sites

despite brood size adjustments (Fig. 1b). Twenty-four and 48 hours after the insecticide applications in 1997, there were no significant differences among sites in percentage of mass gain by chicks nor were there distinct differences in mass changes in adjusted versus non-adjusted nests (Table 4).

Invertebrates

Because aerial invertebrate abundance can be positively correlated with maximum air temperature and season at sites where tree swallows nest in New York state (McCarty 1995), Spearman Rank correlations (Sokal & Rohlf 1981)were performed between number of invertebrates/100 km wind and the date of sample collection and the maximum air temperature on the date of sample collection. Maximum air temperature (MaxAir) and collection date were highly correlated (1996: R= 0.72; p=0.0001; 1997: R=0.62; p=0.003). However, despite strong correlations between these factors they were not consistently related to invertebrate abundance. In 1996 there was no significant correlation between MaxAir and invertebrate density in the orchard. In Control 1 there was a weakly positive correlation between invertebrate density and MaxAir in 1996 (R= 0.44; p=0.008) but we did not find any significant trend in 1997. There was a weakly negative trend between those variables at Control 2 (1996: R=-0.30; p=0.03; 1997: (R= -0.41; p=0.001). These trends were probably significant due to one or two daily high invertebrate densities during the end of the study period at Control 1 and at the end of season sampling at Control 2. There was a weakly positive correlation between collection date of the sample and invertebrate density at the orchard in 1997 (R=-0.28; p=0.03) but not 1996. There was no significant correlation between these variables at either control site in 1996 but in Control 1 there was a weak positive correlation with collection date (R= -0.29; p= 0.03) in 1997. Because MaxAir was significant in several cases, analyses of covariance (ANCOVA) was used to compare invertebrate densities/ kph wind. ANCOVA accounted for the covariates MaxAir and collection date and compared invertebrate density among sites and between nested invertebrate traps within sites. The Tukey's test was used for unequal sample sizes to examine differences in invertebrate densities among sites (Sokal & Rohlf, 1981).

In both years, the orchard had the overall lowest density of invertebrates yet the types of invertebrates caught in the traps were similar among all sites (Table 5 and 6; Fig. 2 and 3). This was most evident during the chick-rearing period in 1996 when the number of days in which there were no invertebrates in the traps at the orchard site was 44.4% versus only 22% at both control sites. Likewise for the same period in 1997, the orchard had 40% of days in which invertebrates were not caught in either trap at the site but there were no days in which traps were empty at Control 2 and this occurred only 1 day at Control 1. Notably, the low invertebrate density at the orchard was persistent and did not coincide with any particular insecticide event (Table 6).

Prior to and during incubation periods in 1996 (13 May-9 June), there were similar types and sizes of invertebrates at all sites (Table 5; Fig. 2a). There were only marginally significant differences in invertebrate density among sites ($p=0.06$) but there were significant differences between trap results within sites ($F^{[3]}=3.2$; $p=0.03$). The orchard had lowest mean density among sites (Table 6).

During pre-incubation and incubation periods in 1997 (19 May-8 June), there were significant differences among sites ($F^{[2]}=8.9$; $p=0.002$) and traps ($F^{[3]}=2.9$; $p=0.04$). The orchard had a significantly lower density of invertebrates than Control 2 and marginally lower in density than Control 1 ($p=0.06$) (Table 6). There were fewer Diptera and more Coleoptera caught at the orchard compared to the control sites where Diptera were the predominant invertebrate (Fig. 3a). The size of the invertebrates caught was not highly variable among sites (Table 5).

During the chick-rearing period in 1996, the size of invertebrates caught varied little among sites (Table 5), however, the Diptera predominated at the control sites while Diptera and Coleoptera were the major components of the orchard samples (Fig. 2b). There were significant differences in invertebrate density among all sites ($F^{[2]}=3.7$; $p=0.03$) with Control 2 having the highest mean density but only marginally higher than both orchard and Control 1($p=0.06$).

The invertebrate abundance was not significantly different among sites or between traps within sites during four sample days immediately prior to the azinphosmethyl spray during

incubation in 1996. After the spray event, there was a significant difference among sites ($F^{[2]}$ =3.7; p= 0.05) with Control 1 showing significantly higher abundance relative to the other sites (Table 6). However, within sites the invertebrate abundance before and after the insecticide showed no significant differences nor was there any differences between traps within sites (Table 6).

During the pre-spray period for the carbaryl application during chick-rearing in 1996, the orchard and Control 2 had similar invertebrate densities while Control 1 had very high abundance (Table 6). After the spray event, the abundance was about the same at all sites (Table 6). During the four sample days in each of the pre- and post-spray periods invertebrate samples were not collected on more than two days at Control 2, therefore, this site was not included in the analysis. The invertebrate abundance was marginally different in the pre-spray period between the orchard and Control 1 ($F^{[1]}$=4.0; p= 0.07) and there were no differences between traps within sites. After the spray event, there were no significant differences between sites or traps. Within sites and between traps, there were no significant differences at any site in invertebrate abundance four days prior versus four days after the spray event (Table 6).

For the azinphosmethyl spray in 1996 during chick-rearing, invertebrate samples were only collected at the orchard site for two of four days after the spray event. Although statistical comparisons among sites could not be made, the density of invertebrates was very low at the orchard before and after the spray (Table 6). Invertebrate abundance was also compared among sites based on the combined results of sample days for each of 48 hours before and 48 hours after azinphosmethyl and carbaryl sprays in 1996. There was only a marginally significant (p=0.07) difference among sites after the sprays and no differences before sprays (Table 6). There were no differences between trap results within sites for those periods.

In 1997 over the entire chick-rearing period (9-23 June), the type and sizes of invertebrates caught were similar among sites (Table 6; Fig.3b) . There were significant differences in invertebrate abundance among sites ($F^{[2]}$=6.8; p=0.001) but not traps within sites. The orchard had the lowest mean density and Control 1 had significantly higher abundance than

either Control 2 or the orchard (Table 6). For the four days before spraying in the chick-rearing period in 1997, there was a highly significant difference in invertebrate abundance among sites ($F^{[2]}$=5.3; p= 0.01) but not between traps within sites. Control 1 and 2 had mean densities which were an order of magnitude above the orchard (Table 6). During four days after the spray event there was a marginally significant difference among sites ($F^{[2]}$=2.8; p= 0.08) with Control 1 having much higher density than either Control 2 or the orchard (Table 6). Within sites, there were no significant differences in invertebrate abundance before and after the insecticide spray event in the orchard (Table 6).

Pesticide Residues

Very little pesticide deposition was found on the filter papers placed in nest-boxes during spray events. In 1996, azinphosmethyl was present on filter papers at concentrations that ranged from non-detectable to 0.0045 ug/cm^2 (N=4 filter papers in separate nest-boxes). Similarly in 1997, 0.091 ug/cm^2 and 0.045 ug/cm^2 azinphosmethyl were found on two filter papers, respectively and 0.074 ug/cm^2 of carbaryl was found on a single filter paper sample.

In our control samples, concentrations of these compounds were non-detectable on filter papers placed in one nest box at each of the control sites during the same period of each spray event at the orchard. Filter papers (N=1 / spray event) which were not exposed to any field site were also submitted for analysis. Concentrations of carbaryl and azinphosmethyl were non-detectable on these blank filter papers.

DISCUSSION

While incubation periods were not different among sites, in the orchard there were significant increases in hunger signalling by tree swallow chicks after OP insecticide applications in both years and, after a second OP spray in 1996, significant decreases in the number of feeding trips by parent birds. Those trends did not occur in the control sites. Despite the changes in behaviour of adults and the low invertebrate density at the orchard, differences in masses of chicks among sites were not related to pesticide use.

Our finding that incubation period did not change after pesticide exposure differs from

two studies that examined nest attentiveness of birds after oral dosing with an OP. In laughing gulls (*Larus atricilla*), 50% inhibition of brain cholinesterase (ChE) was associated with significantly decreased incubation time (White, Mitchell & Hill 1983). Red-winged blackbirds (*Anglais phoeniceus*) decreased nest attendance for the first two hours after dosing with a concentration of methyl-parathion that can induce brain ChE depression of 35-42% (Meyers, Cummings & Bennett 1990). The differences between our findings and those studies may be the degree of pesticide exposures birds experienced. The gull and blackbird studies involved oral dosing at concentrations that can cause severe brain cholinesterase depression whereas our birds were treated with a more diffuse exposure. Our incubating tree swallows often spent 20 min of a 30 min observation period in the nest box where they may have been afforded some protection from the spray. Pesticide concentrations on filter papers in our nest boxes were an order of magnitude lower than those reported by Fleutsch and Sparling (1994) in apple orchards. They placed their filter papers in or near open cup nests. After air blast sprayers applied the chemical, they found a mean concentration of 0.14 ug/cm^2 of OP insecticides on glass fibre filter papers similar to the type used in this study.

Cholinesterase was not measured in the tree swallows in this study because sampling procedure would have upset their behaviour patterns but the decline in feeding trips after the second spray is consistent with the responses found by Burgess *et al* (in press) in this orchard and several nearby orchards. In 1996, birds were exposed to two sprays of azinphosmethyl, a strong cholinesterase-inhibiting OP, as well as carbaryl, a less cholinesterase-inhibiting compound (Smith 1987). In the present study, there were no significant negative trends in feeding trips after the first spray event. But there was a significant decrease in feeding trips in the orchard after the second OP insecticide application. Burgess *et al.* found that tree swallows in this orchard and others in its vicinity did not exhibit significant plasma cholinesterase depression after a single OP insecticide exposure but did experience significant cholinesterase-inhibition after the next OP spray event. Cholinesterase was depressed 41%, on average, and to a maximum of 64-71% in one pair after the second OP exposure (Burgess *et al.* submitted). The

lack of significant change on feeding trips in 1997 after only one azinphosmethyl exposure supports this hypothesis although we cannot discount the possibility that small sample sizes of nests in both years biased the results. Similarly, Patnode and White (1991) did not find any significant change in the behaviours of either northern cardinal (*Cardinalis cardinalis*) or northern mockingbirds (*Mimus polyglottos*) in pecan & peanut orchards after exposure to a single combined OP/CARB spray event.

Reductions in feeding trips by tree swallows in our study were also predicted based on the significant decline in feeding trips taken by wild, OP-dosed starlings in which 50% brain cholinesterase inhibition was induced (Grue, Powell & McChesney 1983) although the effect in starlings occurred after only one OP dosing. The dose induced such severe brain ChE depression that an immediate behavioural response is not surprising. However, in contrast to our hypothesis of a more severe effect with two OP sprays versus a single spray, neurotoxic effects were more severe in songbirds after the first exposure to fenitrothion sprays over forests compared to a subsequent spray but this was likely due to the removal of highly exposed or sensitive birds in the first spray event (Busby, Pearce, Garrity & Reynolds 1983). Holmes and Boag (1990) did show that significant brain cholinesterase depression in zebra finches (*Taeniopygia guttata*) can persist for up to ten days after a single oral OP exposure of 1.04 mg/kg. Hence, a second exposure in the orchard within this period might suppress ChE even further. This scenario did occur in our study in 1996 in which a carbaryl spray followed the first OP spray within a week and then another OP spray followed a week after that.

In contrast to the incubation results, the effects on feeding trips also suggest tree swallows may be more sensitive to insecticide use than some other bird species. In controlled settings of oral dosings which induce significant brain cholinesterase inhibition, American kestrels (*Falco sparverius*) show no alterations on prey-capturing ability when dosed with the OP acephate (Rudolph *et al.* 1984) and black-capped chickadees (*Parus atricapillus*) do not decrease their food cacheing and retrieval abilities when treated with fenitrothion (Mineau, Boag & Beninger 1994).

There was little evidence to support the alternate hypothesis that insecticide removal of invertebrate resources influenced adult feeding behaviour. Invertebrate abundance was consistently lowest in the orchard but this did not coincide with spray events even when weather and season were accounted for. Hussell (1988) found that the number of feeding trips by parent tree swallows is unaffected by chronic or twenty-four hour reductions in insect abundance. Therefore, if a change in food abundance after insecticide spray events had occurred and the sampling methods had failed to detect this effect, then a change in feeding trips by adults would not be expected yet there was a significant change in the orchard. Furthermore, there were no significant changes in behaviours of birds after non-OP spray events during incubation and chick-rearing which suggests disturbance was not a factor.

In contrast to the adults, chick behaviour was not consistent with known symptoms of cholinesterase inhibition. Although adult starlings can display brief periods of hyperactivity within hours after OP exposure, Hart (1983) found that they significantly reduced their activity and singing in a 24 hour period after OP dosing. Hunger signalling in tree swallow chicks increased after OP/CARB sprays in both years of this study. This is also consistent with results reported by Burgess *et al* (in press) that showed tree swallow chicks had only marginal ChE depression after OP spray events in this orchard and several others when compared to control birds. Since pesticides were found in the nest boxes it is possible that some exposure occurred dermally on the chicks, especially when they were young and unfeathered and/or exposure may have occurred by inhalation. If this occurred, it appears it was not severe enough to have any subduing effect on behaviour as expected in cholinesterase suppression. Studies also indicate that contaminated food was unlikely to be an important source of exposure. After a spray of the OP insecticide fenthion on a marsh, Powell (1984) found the chemical on sweep-sampled invertebrates but not on food delivered to wild nestling red-winged blackbirds. Tree swallows are also unlikely to deliver contaminated food to their young because dead and dying insects would be on or near the ground and swallows feed exclusively on aerial invertebrates during chick-rearing (Robertson, Stutchbury & Cohen 1992)

The more probable explanations for increased calling time is that food quantity was persistently low in the orchard or the amount of food delivered per feeding trip was small. Besides the many days in which their were no invertebrates in traps at the orchard, the average densities of invertebrates at the orchard were lower than at control sites and almost an order of magnitude lower in density compared to Quinney's 'high-food' site sampled in May and June with the same techniques (Quinney, Hussell & Ankney 1986). Hussell (1988) found that calling time of tree swallow chicks increased during persistently low food resources but no increase in calling occurred in response to short-term (<24 hours) decreases in food. In both years, calling time increased during the entire chick-rearing period and over the three to five days after the OP spray events. Hussell also found mass delivery per feeding sortie by tree swallow parents declines during short-term reductions in food abundance (Hussell 1988). Although the types and sizes of invertebrates were similar among the sites and similar between incubation and chick-rearing periods within sites there may have been an effect on the mass of food delivered per feeding trip that our sampling methods were not designed to detect.

Despite the increased chick calling and the decreases in feeding trips by parents, ultimately, nestling masses at the orchard were unaffected. Any change in food quality or quantity and parental behaviour was not extreme or prolonged enough to have any significant effects. Since energy requirements for chick begging are low in tree swallows (McCarty 1996), an increase in calling time in orchard birds would not be expected to decrease their mass. The paucity of an effect may be related to alternate feeding sources. Tree swallows were observed feeding in or above the orchard as well as outside of it. Birds also fed near the orchard but over ponds located within 250 m of the nesting area. Perhaps when insect abundance was unusually low in the orchard these birds were able to compensate by feeding nearby allowing them to provide enough food for chicks to grow normally.

These findings contrast with those of Grue, Powell & McChesney (1982) in which nestlings of OP -treated starling females gained significantly less mass than controls. Patnode and White (1991) also found significantly lower nestling masses in songbirds reared in orchards.

In their study four OP applications were made during the study whereas our tree swallows were only exposed to a maximum of two OP events. Our results are comparable to the lack of effects on nestling masses when red-winged blackbird parents are exposed to a single OP dose in the wild (Powell 1984; Meyers, Cummings & Bennett 1990), and when black-throated blue warblers (*Dendroica caerulescens*) rear chicks in forests sprayed once with *Bacillus thuringensis* (Rodenhouse and Holmes 1992). Hart (1983) suggests that wild birds show less effects in the wild because they overcome the effects of ChE depression in stress situations such as exposure to a predator or, in this study, the constantly increasing demand for food by nestlings.

Definitive statements about the impact of insecticides on tree swallows nesting in orchards cannot be made since cholinesterase was not measured in these birds. Also, orchard exposures were not replicated because spray schedules differ among farms to the extent that exact replications of chemical mixtures and time of exposures would be impossible. However, the spray schedules used in this orchard are generally representative of the types of compounds and chronology of spray events of orchards in southern Ontario (OMAFR 1996). It appears that food resources are consistently low and adult birds are sensitive to OP spray events during the critical chick-rearing period. They are able to compensate and feed chicks adequately even when brood size is artificially increased possibly due to nearby feeding habitats that are unaffected by pesticides. This suggests a need for maintaining relatively natural habitats near to agricultural areas if songbirds nesting in these landscapes are to be successful. The implications of this finding are important because the survival of tree swallow chicks in their first year is dependent on their mass at fledging (McCarty 1995).

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Table 1. Behaviour monitoring periods for all sites and spray schedule for orchard (1996,1997)

Year	Spray No.	Status of Nests	Compound	Application Rate	Pre-spray monitoring periods (hours); [dates]	Spray Date	Post-spray period (hours)
1996	1	Clutches not complete	Mancozeb Myclobutanil Cypermethrin	3 kg/ha 2.5 kg/ha 250 ml/ha	NA	21 May	NA
1996	2	Incubation	Mancozeb	3 kg/ha	(24h; 7h; 1h) [29-30 May]	30 May	(1.5h; 15h; 24h; 48h) [30-31 May; 1 June]
1996	3	Incubation	Azinphosmethyl Calcium chelate	2 kg/ha 5 l/ha	(48h; 24h) [4-5 June]	6 June	(1.5h;3h;9h;24h;29h;50h) [6-8 June]
1996	4	Chick-rearing	Glyphosate	1 kg /ha	NA	8 June	NA
1996	5	Chick-rearing	Mancozeb Myclobutanil	3 kg/ha 4.35 kg/ha	NA NA	10 June	NA NA
1996	6	Chick-rearing	Carbaryl	1.5 kg/ha	(110h; 96h;72h;48h) [10-13 June]	14 June	(4h;8.5h;24h;35h;72h;96h;110h) [14-19 June]
1996	7	Chick-rearing	Azinphosmethyl Mancozeb Calcium chelate Phosphorus	2 kg/ha 3 kg/ha 5 l/ha 1 l/ha	(72h;60h;12h) [14-19 June]	20 June	(5h;10h;24h;30h;48h) [20-22 June]

NA= Not applicable because monitoring was not conducted for these sprays

Table 1 (CONTINUED). Behaviour monitoring periods for all sites and spray schedule for orchard (1996,1997)

Year	Spray No.	Status of Nests	Compound	Application Rate	Pre-spray monitoring periods (hours) [dates]	Spray Date	Post-spray period (hours) [dates]
1997	1	Incubation	Mancozeb Myclobutanil Cypermethrin	3 kg/ha 2.5 kg/ha 250 ml/ha	NA	24 May	NA
1997	2	Incubation	Mancozeb Myclobutanil	3 kg/ha 2.5 kg/ha	NA	3 June	NA
1997	3	Chick-rearing	Azinphosmethyl	2 kg/ha	(6d;5d;96h;72h;24h;1h)	15 June am	(6h;24h;48h;72h;96h;5-7d)
1997	4	Chick-rearing	Carbaryl	1.5 kg/ha	[9-15 June]	15 June pm	[15.5-18 June; 19-23 June]

NA= Not applicable because monitoring was not conducted for these sprays

Table 2. Total incubation period to hatching and incubation time observed during 30 minute intervals in two non-sprayed sites and one insecticide sprayed apple orchard (1996)

Mean (SD) incubation period(days) from the first day of incubation to hatch day +/-12 hours		
Orchard	Control Site 1	Control Site 2
13.8 (0.6)	14.6(0.7)	13.6(0.9)
N=6 nests	N=6 nests	N=6 nests

A, B= Different letters signify significant difference among sites for this parameter within the observation period ($p<=0.05$). Where letters do not appear there were no significant differences among sites.

Table 2(CONTINUED). Total incubation period to hatching and incubation time observed during 30 minute intervals in two non-sprayed sites and one insecticide sprayed apple orchard (1996)

Mean (SD) Incubation Time (minutes) per 30 minute observation period

	Orchard	Control Site 1	Control Site 2
Pre-Fungicide	N=6	N=6	N=6
24 hrs	20.4 (12.2)	19.0 (9.3)	21.6 (6.3)
7 hrs	23.1 (5.8)	25.3 (3.7)	18.5 (5.3)
1 hrs	21.0 (8.5)	18.8 (5.4)	22.8 (7.1)
Mean *	21.5 (1.2)	20.6 (2.3)	20.9 (2.8)
Post-Fungicide			
1.5 hrs	15.4 (7.1)	21.3 (6.4)	15.3(2.4)
15 hrs	23.6 (4.2)	18.3 (6.7)	23.4(3.7)
24 hrs	18.4 (5.8)	16.5(4.7)	20.3(5.9)
48 hrs	12.7(3.7)	13.8(3.6)	22.6(1.5)

* Mean Incubation Time of 3 observation periods during 24 hrs prior to Fungicide Spray

Table 2(CONTINUED). Total incubation period to hatching and incubation time observed during 30 minute intervals in two non-sprayed sites and one insecticide sprayed apple orchard (1996)

Mean (SD) Incubation Time (minutes) per 30 minute observation period

	Orchard	Control Site 1	Control Site 2
Pre-OP Insecticide			
72 hrs	20.5 (6.1)	20.1(3.1)	17.5 (5.1)
24 hrs	17.8 (4.8)	20.1 (6.1)	20.1 (6.9)
Mean **	19.1(1.4)	20.1(0)	18.8(1.3)
Post-OP Insecticide (hours)	N=4	N=3	N=4
1.5	23(3.5) A	23(3.0) A	15.5(2.7) B
3	20.3(3.9)	21.7(2.1)	19.5(0.5)
6	22.0(0.7)	24.3(3.7)	21.8(3.7)
9	26.8(3.1)	25.3(2.2)	20.3(5.8)
24	20.2(7.9)	23.8(5.0)	22.8(7.2)
29	21.0(6.9)	22.5(0.9)	13.0(1.2)
50	17.3(8.2)	23.5(0.5)	24.0(4.5)

A, B= Different letters signify significant difference among sites for this parameter within the observation period ($p<=0.05$). Where letters do not appear there were no significant differences among sites.

** Mean Incubation Time of 2 observation periods during 72 hrs prior to OP insecticide

Table 3a. Mean Spearman rank correlation coefficients (R) for tree swallow behaviour relative to age of chicks in an apple orchard and two non-sprayed sites in southern Ontario (1996)

Behaviour	Site		Overall monitoring period (observations) (10-22 June) R value	Overall monitoring period (taped behaviours) * (17-22 June) R value
Feeding trips/ 30 min. observation	Orchard		0.10	-
	Control 1		***0.48***	-
	Control 2		***0.42***	-
Feeding Trips/Sec. of tape after first feeding trip	Orchard		-	-0.19
	Control 1		-	***0.56***
	Control 2		-	0:39 **
Calling time/Sec	Orchard	CL1/s	-	***0.45***
		CL2/s	-	***0.40***
	Control 1	CL1/s	-	***0.61***
		CL2/s	-	***0.58***
	Control 2	CL1/s	-	***0.61***
		CL2/s	-	***0.59***
Time in Box (male + female)	Orchard		***-0.67***	-
	Control 1		***-0.56***	-
	Control 2		***-0.69***	-

Bold-Italic R Values are significant at p<=0.0125 based on sequential Bonferroni test

* 17-22 June only; prior to 17 June chicks were too young to use tape recorders in nestboxes

** marginally significant p=0.02-0.05

- = no data collected during this period

CL1/s= calling time / second after the first chick calls heard on tape

CL2/s= calling time / second after the first feeding trip by adult heard on tape

Table 3a (CONTINUED). Mean Spearman rank correlation coefficients (R) for tree swallow relative to age of chicks in an apple orchard and two non-sprayed sites in southern Ontario (1996)

Behaviour	Site		4 days Pre-Carbaryl (Spray 6) (10-13 June) R value	Pre-azinphosmethyl (Spray 7) & post-carbaryl * (14-19 June) R value	5 h to 48 h Post-azinphosmethyl (Spray 7) (20-22 June) R value
Feeding trips/ 30 min. observation	Orchard		-0.83 **	0.05	***-0.37***
	Control 1		-0.28	***0.49***	-0.41
	Control 2		-	-0.11	0.20
Feeding Trips/Sec. of tape after first feedin trip	Orchard		-	0.34	***-0.36***
	Control 1		-	0.29	0.31
	Control 2		-	0.35	0.25 ***
Calling time/Sec	Orchard	CL1/s	-	0.29	***0.37***
		CL2/s	-	***0.79***	***0.38***
	Control 1	CL1/s	-	0.23	0.38
		CL2/s	-	0.09	0.34
	Control 2	CL1/s	-	0.31	0.32
		CL2/s	-	***0.74***	0.29
Time in Box (male + female)	Orchard		-	***-0.63***	-0.35
	Control 1		-	-0.37	-0.25
	Control 2		-	***-0.3***	-0.21

Bold-Italic R Values are significant at p<=0.0125 based on sequential Bonferroni test

* 14-19 June for observations; 17-19 June for taped behaviour monitoring. For taped observations this applies to 3 to 5 days post-carbaryl and 3 days before azinphosmethyl (17-19 June); for 30 min.field observations this applies to 0.5 to 5 days post-carbaryl and 5 days prior to azinphosmethyl spray (14-19 June).

** N=2; *** marginally significant p=0.02-0.05; - = no data collected during this period

CL1/s= calling time / second after the first chick calls heard on tape

CL2/s= calling time / second after the first feeding trip by adult heard on tape

Table 3b. Mean Spearman rank correlation coefficients (R) for tree swallow behaviour relative to age of chicks in an apple orchard and two non-sprayed sites in southern Ontario (1997)

Behaviour	Site		Overall Monitoring period (9-23 June) R value	6.5 days pre-insecticide (Spray 3 & 4) (9-15 June) R value	0.5-3.0 days post-insecticides (15-18 June) R value	4-7 days post-insecticides (19-23 June) R value
Feeding trips/ 30 min. observation	Orchard		***0.60***	***0.47***	0.06	***0.54***
	Control 1		***0.68***	***0.68***	0.24	0.33
	Control 2		0.20	0.29	-0.04	0.19
Feeding Trips/Sec. of tape after first feeding trip	Orchard		0.11	-	-0.14	0.58
	Control 1		***0.38***	0.17	-0.37	-0.05
	Control 2		***0.64***	-	0.35	-
Calling time/Sec	Orchard	CL1/s	***0.93***	-	***0.81***	***0.85***
		CL2/s	***0.89***	-	0.75	***0.79***
	Control 1	CL1/s	***0.68***	0.16	0.39	0.29
		CL2/s	***0.7***	0.19	0.36	0.15
	Control 2	CL1/s	***0.84***	-	***0.75***	0.4
		CL2/s	***0.87***	-	0.71	0.8
Time in Box (male + female)	Orchard	NA	***-0.72***	-0.47	***-0.68***	0.23
	Control 1	NA	***-0.65***	-0.25	***-0.66***	0.18
	Control 2	NA	***-0.66***	-0.19	-0.41	-0.54

Bold-Italic R Values are significant at p<=0.0125 based on sequential Bonferroni test
***** marginally significant p=0.07
- = N < 3 nests monitored therefore correlations not performed
CL1/s= calling time / second after the first chick calls heard on tape
CL2/s= calling time / second after the first feeding trip by adult heard on tape

Table 4. Mean (Standard Deviation) of percentage change in weights of chicks after spray events (1996, 1997)

Year	Spray Event	Compound	Site	N nests	Nest Type	Percentage change in weights of chicks post-Spray: 24 hrs *	48 hrs *
1996	6	Carbaryl	Orchard	6	All	39.1(11.0)	49.7(12.3)
				4	Not adjusted	43.6(9.4)	53.7(10.4)
				2	Adjusted	30.1(9.6)	41.8(15.4)
		No spray applied	Control 1	5	All	40.4(26.2)	58.1(8.2)
				2	Not adjusted	69.7(0)	83.0(0)
				3	Adjusted	25.8(9.4)	46.9(27.0)
		No spray applied	Control 2	6	All	24.1(12.1)	35.2(10.2)
				3	Not adjusted	20.2(0)	36.9(0)
				3	Adjusted	26.1(16.4)	34.3(14.3)
1996	7	Azinphosmethyl	Orchard	6	All	3.5(3.1)	2.4(2.7)
		Mancozeb	Orchard	2	Not adjusted	4.5(3.3)	2.7(3.2)
		Calcium chelate Phosphorus	Orchard	4	Adjusted	1.5 (1.4)	1.5(0)
		No spray applied	Control 1	6	All	6.2(4.1)	6.9(5.2)
			Control 1	3	Not adjusted	6.8(4.6)	9.1(4.6)
			Control 1	3	Adjusted	5.8(4.7)	5.5(7.2)
		No spray applied	Control 2	6	All	3.9(3.1)	4.5(5.2)
			Control 2	3	Not adjusted	3.6(0.9)	3.7(3.8)
			Control 2	3	Adjusted	4.3(4.8)	5.3(7.3)

* Statistical analysis of % change in total nests/ site did not find any significant differences for any spray event or time period ($p<=0.05$)

Table 4 (CONTINUED). Mean (Standard Deviation) of percentage change in weights of chicks after spray events (1996, 1997)

Year	Spray Event	Compound	Site	N nests	Nest Type	Percentage change in weights of chicks post-Spray: 24 hrs *	48 hrs *
1997	3	Azinphosmethyl	Orchard	6	All	38.4(6.9)	63.3(38.7)
		Carbaryl	Orchard	5	Not adjusted	37.5(7.0)	74(35.5)
			Orchard	1	Adjusted	42.0	21.0
		Water	Control 1	3	All	25.2(11.2)	29.5(16.5)
			Control 1	1	Not adjusted	16.0	21.0
			Control 1	2	Adjusted	28.0(11.0)	32.3(19.0)
		Water	Control 2	2	All	23.5(7.7)	43.4(9.2)
			Control 2	2	Not adjusted	23.5(7.7)	43.4(9.2)
			Control 2	0	Adjusted	-	-

* Statistical analysis of % change in total nests/ site did not find any significant differences for any spray event or time period ($p<=0.05$)

Table 5. Size of invertebrates caught in traps in orchard and two non-sprayed site (1996, 1997)

Year	Status of Nests	Site	N	Percentage of invertebrates in each Size Category: 0.1-3.4mm	3.5-6mm	>6 mm
1996	Incubation (13 May-9 June)	Orchard	104	74.0	16.4	9.6
		Control 1	280	63.5	33.3	3.1
		Control 2	63	90.7	6.7	2.6
1996	Chick-rearing (10-22 June)	Orchard	7	71.0	29.6	0
		Control 1	25	84.0	16.0	0
		Control 2	4	25.0	75.0	0
1997	Incubation (19 May-8 June)	Orchard	80	60.0	35.0	4.0
		Control 1	161	57.0	23.0	20.0
		Control 2	936	90.8	8.1	1.1
1997	Chick-rearing (9-23 June)	Orchard	29	66.0	24.0	10.0
		Control 1	44	85.8	13.4	0.8
		Control 2	120	79.6	13.6	6.8

N= total number of invertebrates caught

Table 6. Mean (Standard Deviation) number of Invertebrates per 100 km wind at two non-sprayed sites and one insecticide sprayed apple orchard (1996, 1997)

Year	Spray No.	Status of Nests	Sample Period	No. days sampled/ site	Orchard	Control 1	Control 2
1996	NA	Inc./Chick	**Entire Study Period 13 May-23 June**	28,27 *	3.6 (8.6) **C**	9.9 (22.8) **C**	15.5 (47.8) **C**
1997	NA	Inc./Chick	**Entire Study Period 19 May- 21 June**	30,31,32 **	3.6 (5.8) **D**	48.0 (88.6) **C**	37.5 (63.2) **C**
1996	NA	Inc.	**Pre-Incubation & Incubation Period 13 May-9 June**	19	4.9(10.2) C ***	6.6(15.5) C ***	21.4(55.8) C ***
1997	NA	Inc.	**Pre-Incubation & Incubation Period 19 May-8 June**	21	4.2 (6.9) C***	42.5(72.9) **D**	79.1(113.8) C ***

Letters (A or B) on the same line as the mean (st. dev.) signify differences between pre- and post-spray results within each site ($p<=0.05$)
Bold letters (C or D) underneath the mean (st.error) values for each site signify differences among sites for that study period ($p<=0.05$)
* Orchard and Control 2 sampled 28 days; Control 1 sampled 27 days
** Control 2 sampled 30 days; Control 1 sampled 31 days; Orchard sampled 32 days
*** Marginally significant $p=0.07$
NA = Not applicable for this parameter
Inc.= Incubation; Chick= chick-rearing

Table 6 (CONTINUED). Mean (Standard Deviation) number of invertebrates per 100 km wind at two non-sprayed sites and one insecticide sprayed apple orchard (1996, 1997)

Year	Spray No.	Status of Nests	Sample Period	No. days sampled/ site	Orchard	Control 1	Control 2
1996	NA	Chick	**Chick-rearing Period 10-21 June**	10	0.98 (1.5) **C** *	16.9(33.3) **C** *	0.95(1.6) **C** *
1997	NA		**Chick-rearing Period 9-23 June**	15	2.5(4.0) **C**	30.0(13.2) **D**	30.0(29.4) **C**
1996	NA	Inc./Chick	**Combined pre-spray****	2	0.25(0.6) A **C**	2.0(3.5) A **C**	41.1(104.5) **C**
1996	NA	Inc./Chick	**Combined post- spray ****	2	1.0(1.6) A **C** *	7.1(10.9)A **C** *	2.1(2.6) A **C** *

Letters (A or B) on the same line as the mean (st. dev.) signify differences between pre- and post-spray results within each site ($p<=0.05$)
Bold letters (C or D) underneath the mean (st.error) values for each site signify differences among sites for that study period ($p<=0.05$)
* marginally significant $p=0.07$
** Comparing the combined results for 48 hrs prior to spray to the combined results for 48 hrs post-spray for three insecticide spray events (two azinphosmethyl, one carbaryl)
NA = Not applicable for this parameter
Inc.= incubation; Chick= chick-rearing

Table 6 (CONTINUED). Mean (Standard Deviation) number of Invertebrates per 100 km wind at two non-sprayed sites and one insecticide sprayed apple orchard (1996, 1997)

Year	Spray No.	Status of Nests	Sample Period	No. days sampled/ site	Orchard	Control 1	Control 2
1996	3	Inc.	**OP Insecticide**				
			Pre-spray (31 May-5 June	4	1.8(2.9) A C	16.4(27.4)A C	3.7(6.8) A C
			Post-spray (6 -11 June)	4	0.7(1.3) A C	35.0(44.6) A D	3.4(5.1) A C
1996	4	Chick	**Carbamate Insecticide**				
			Pre-spray (10-13 June)	4	1.0(1.4) A C *	30.9(45.8) A C *	0.7 (2.1)
			Post-spray (14-18 June)	4	1.1(1.7) A C	1.7(3.9) A C	0.7 (0.7)
1996	5	Chick	**OP Insecticide** **				
			Pre-spray (18, 19 June)	2	0	0	0
			Post-spray (20,21 June)	2	1.7 (1.7)	0.8(0.8)	7.8(1.0)
1997	1	Chick	**OP Insecticide & Carbamate Insecticide**				
			Pre-spray	4	0.53(1.0) A D	10.6(15.2) A C	19.(10.7) A C
			Post-spray	4	0.73(1.4) A C *	53.6(77.2) A C *	14.5(14.3) A C *

Letters (A or B) on the same line as the mean (st. dev.) signify differences between pre- and post-spray results within each site ($p<=0.05$)
Bold letters (C or D) underneath the mean (st.error) values for each site signify differences among sites for that study period ($p<=0.05$)

* Marginally significant p=0.07
*** Because comparisons were made for 48 hours pre- and post-azinphos methyl spray during chick-rearing in 1996 our sample size of sampling dates (N=2 days) was too small for each period for statistical analysis
NA = Not applicable for this parameter
Inc.= Incubation; Chick= chick-rearing

Appendix 1. Table 1.Range (minimum, mode, maximum) in nest characteristics at two non-sprayed sites and one orchard (1996,1997)

		Incubation						
		1996						
		(min., mode, max.)				No. of Females with Ages:		
Site	No. Nests	Unadjusted Clutch Size *	No. feathers/ nest	Incubation period (days)	First Day of Incubation	Second year	After Hatch Year	After Second Year
Orchard	6	5,5,7	20,20,25	13,14,15	24-26 May	1	0	5
Control 1	6	6,6,6	5,20,25	12,14,15	24-26 May	0	3	3
Control 2	6	5,6,7	5,20,25	13,15,15	24-26 May	5	0	1

* Clutches adjusted to 5 eggs in 1996

Appendix 1. Table 1.(CONTINUED) Range (minimum, mode, maximum) in nest characteristics at two non-sprayed sites and one orchard (1996,1997)

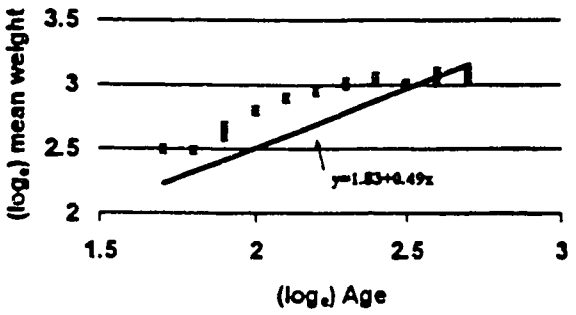
	Chick-Rearing							
	1996		1997					
		(min., mode, max.)		(min., mode, max.)		No. of Females with Ages:		
Site	No. Nests	Unadjusted Brood Size **	No. Nests	Unadjusted Brood Size **	No. feathers/ nest	Second year	After Hatch Year	After Hatch Year
Orchard	6	5,5,7	5	6,6,7	15,20,20	0	1	4
Control 1	6	4,6,6	5	5,6,6	15,20,25	0	0	5
Control 2	6	5,6,7	3	6,6,6	15,25, 25	0	0	3

** Broods adjusted to 5 young in 1996; adjusted to 6 young in 1997

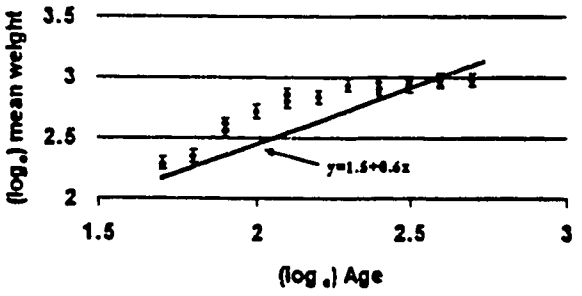
Figure 1. Mean weights of chicks and linear relationships between age and mean weights at one apple orchard and two non-sprayed sites in (a)1996 and (b) 1997.

a. 1996

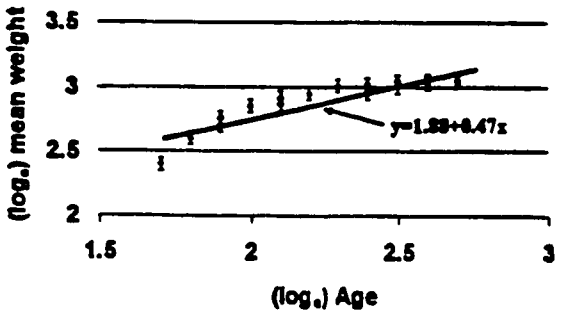
Orchard



Control 1

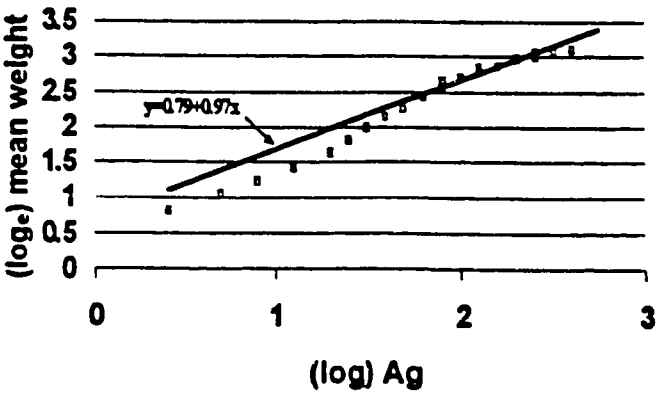


Control 2

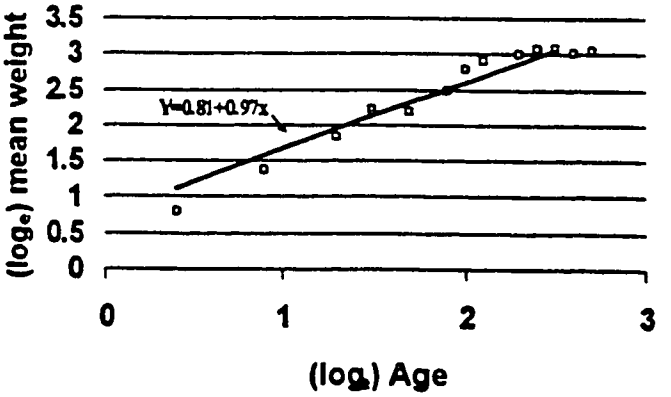


b.1997

Orchard



Control 1



Control 2

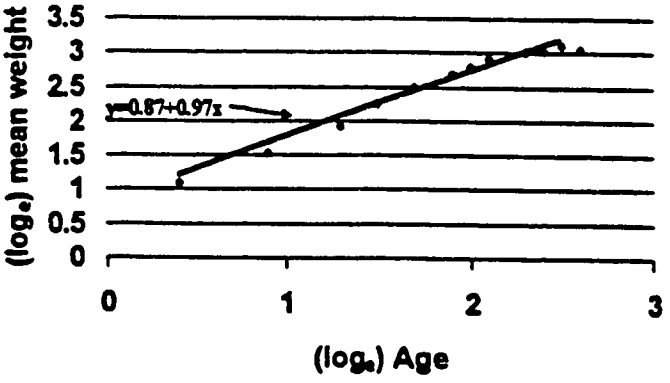
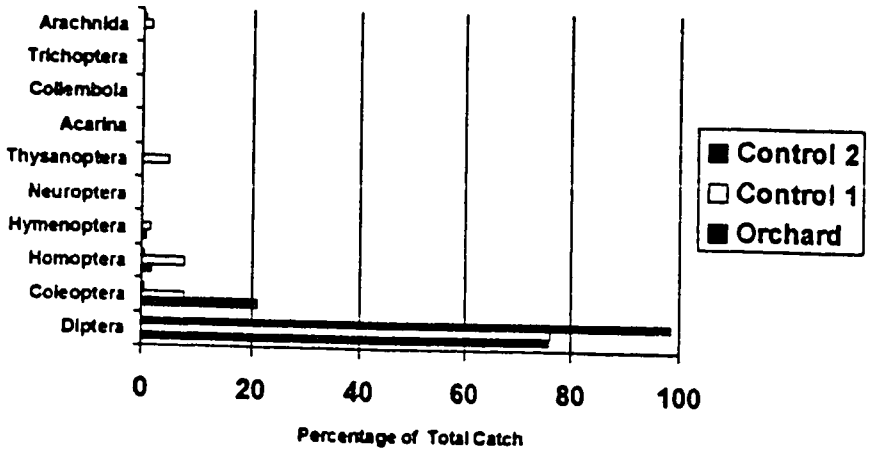


Figure 2 . Types and relative catch of invertebrates in traps at one orchard and two non-sprayed sites during (a) incubation and (b) chick-rearing in 1996.

a. Incubation Period



b. Chick-rearing period

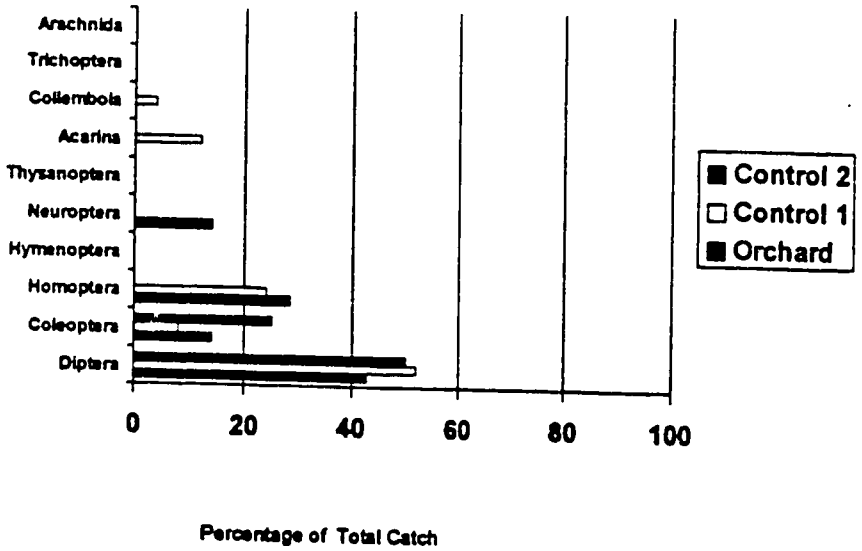
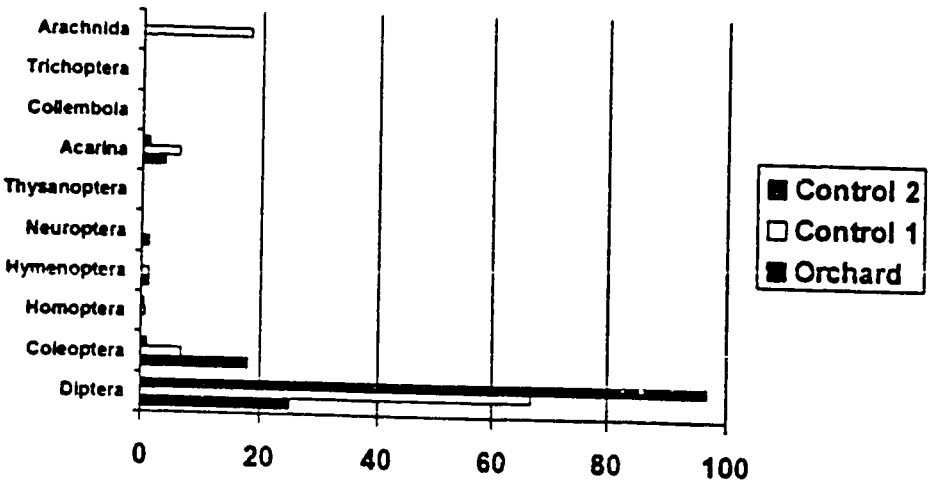
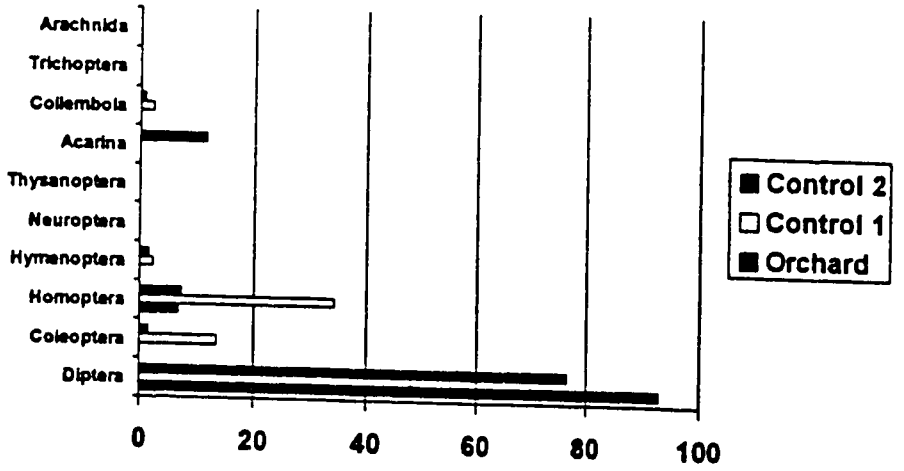


Figure 3 . Types and relative catch of invertebrates in traps at one orchard and two non-sprayed sites during (a) incubation and (b) chick-rearing in 1997.

a. Incubation Period



b. Chick-rearing Period



Chapter Four.

Reproduction of Cavity-Nesting Birds in Pesticide-Sprayed Apple Orchards in Southern Ontario, Canada (1988-1994)

Reproduction of Cavity-Nesting Birds in Pesticide-Sprayed Apple Orchards in Southern Ontario, Canada (1988-1994)

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ABSTRACT

Egg and chick survival and pesticide exposure of tree swallows (*Tachycineta bicolor*) and eastern bluebirds (*Sialia sialis*) were monitored annually using nest boxes in sprayed and non-sprayed apple orchards in southern Ontario during 1988-1994. We examined the associations between reproductive rates and organochlorine residues in eggs and the degree of exposure and toxicity of pesticides applied during the study period. Because many pesticides in current use are not persistent in wildlife tissues, we developed a toxicity score to describe the exposure for each nest. The toxicity score was calculated as the product of the extent of the orchard sprayed and the application rate of the chemicals divided by an acute reproductive toxicity index of each chemical.

We found total organochlorine concentrations in tree swallow eggs ranged from 0.74 to 3.5 ug/g and in eastern bluebird eggs ranged from 0.47 to 106.3 ug/g wet wt. There was a significant increase in unhatched eggs in eastern bluebirds as organochlorine concentrations increased in eggs. At the gradient of contamination found in tree swallow eggs, there were no trends between reproductive rates and organochlorine levels.

In more than half the study years and over the entire study period, egg fertility and daily survival rates of eggs and chicks of tree swallows declined with increased toxicity scores of pesticides used during 1988-1994. Fewer years and reproductive parameters were affected in eastern bluebirds. Reduced egg fertility was detected in eastern bluebirds as toxicity scores increased but this only occurred in two years and there was no overall trend for 1988-1994. Daily egg and chick survival was not associated with pesticide exposure in the clutches of eastern bluebirds initiated prior to 1st June in each year. The bluebird nests initiated after that date had significantly lower daily chick or egg survival rates as pesticide exposure and toxicity increased in four study years. We cannot discount the possibility that organochlorine chemicals contributed to these effects in bluebirds. For the compounds sprayed during 1988-1994, acute toxicity indices were highest for organophosphorus (OP) and carbamate (CARB) pesticides, oil, sulfur and Carzol whereas other pesticides and minerals applied to orchards had very low acute toxicities. We suspect the OP and CARB compounds were most responsible for lower reproduction in tree swallows and this is consistent with findings of other studies. In North America, the reproduction of six passerine species have now been shown to be negatively impacted by pesticide use in orchards.This is of growing concern because continual loss of natural habitats increases the likelihood that birds will utilize semi-natural agricultural areas such as orchards.

INTRODUCTION

Apple orchards are used by many passerine species for feeding and nesting (Graham and Desgranges, 1993; Fleutsch and Sparling 1994) but pesticide applications in orchards, particularly cholinesterase-inhibiting insecticides, have been implicated in lowered reproductive success in some bird species that nest there (Patnode and White, 1991; Fleutsch and Sparling 1994). These effects and other sublethal health impacts on birds in orchards are of particular concern due to the continual loss of natural habitats which increases the likelihood that birds will utilize semi-natural agricultural areas (Graham and Desgranges, 1993) such as fruit orchards.

Although organophosphorus (OP) and carbamate (CARB) insecticides have been sprayed in orchards for decades, only recently have researchers applied more subtle analyses that examine daily survival rates of eggs and chicks relative to the toxicity of individual pesticides to birds (Patnode and White, 1991; Fleutsch and Sparling 1994). Where decreased egg and chick survival was found, the compounds of greatest toxicity to reproduction appeared to be the OP and CARB chemicals. However, some studies have reported no effect of OPs on avian reproduction in orchards (Hardy et al., 1993). Unfortunately, the potential effects on reproduction of residual organochlorine chemicals (OCs) have not been integrated into studies examining the impact of in-use pesticides despite the high OC concentrations in soils and wildlife in orchards (Johnson et al., 1976; Blus et al., 1987; Hebert et al 1994; Elliott et al., 1994) and the known toxicity of OCs to avian reproduction (Jefferies 1967; Keith and Mitchell 1993). Studies on the reproduction of birds in orchards have also been limited to short-term studies of less than three years which limits the interpretation of long-term effects and inter-year differences in effects.

The purpose of our study was to extend the understanding of the effects of pesticides on reproductive success of birds nesting in pesticide-sprayed apple orchards. We measured the reproduction of tree swallows (*Tachycineta bicolor*) and eastern bluebirds (*Sialia sialis*) in sprayed apple orchards in relation to the degree of their exposure to in-use pesticides and the toxicity of each of those compounds to avian reproduction and in relation to their

bioaccumulation of persistent organochlorine residues in eggs. Our analysis examines these trends for a seven year period (1988-1994) in southern Ontario.

METHODS

Study Areas and Nest Monitoring

Twenty four sites were studied during 1988-1994. Nineteen sites were conventionally managed and sprayed apple orchards, one site was an organic orchard which was sprayed but not with chemical pesticides other than sulfur, and four sites were rural locations which were either pastures where pesticides were never sprayed or orchards that were never sprayed or mowed during our study period. For all but two of the conventionally managed orchards there was at least one year that nests in each orchard were not sprayed. All sites were located in the Great Lakes basin in southern Ontario, Canada (approximate Latitude 43°15'N/ Longitude 80°2'W).

Sprayed orchards contained a mix of standard-size and semi-dwarf apple trees and were mowed once a month. Aerial broadcast sprayers were used to apply all chemicals except herbicides which were applied with ground level boom sprayers. The spray schedules, chemical application rates and the approximate area of orchard sprayed on each occasion of pesticide application were provided by the orchard owners for each year of the study (Table 1) and these were confirmed with personal observations.

Eastern bluebirds and tree swallows nest in cavities and to standardize exposure and nesting conditions among study sites, we installed nest boxes which these species readily occupied. Each nest box was 8-10m from its nearest neighbour and entrances to all nest boxes faced south to avoid the prevailing wind. We deterred nest predators by applying a small amount of axle grease to nest poles a week prior to the start of breeding season. In the orchards, nest boxes were located between apple trees. All sites had nest boxes installed at least six months prior to the breeding season. Each year we monitored nests from mid-April to mid-August. In

1988-1990 and 1994, nests were checked every third day during the egg laying and every other day during hatching periods, whereas during incubation and chick-rearing, nests were checked every four days. In 1991-1993, nests were checked once every seven to ten days throughout the egg-laying and chick-rearing periods.

During each nest check, we recorded the status of the nest contents. On each nest visit the number of eggs, nestlings (live or dead) and fledged young were noted. Active nests were visited until the fates of all offspring were determined. Eggs that had not hatched once chicks in that brood were older than five days were considered unhatched eggs. The number of unhatched eggs was counted and many but not all unhatched eggs were opened to determine their developmental status. These were categorized as infertile and/or lacking visible embryonic development(<7 days) or containing visible embryos at seven to ten days or greater than ten days of age. Eggs that were missing from the clutch were also noted. The first time nestlings were found in nests their ages were estimated using established developmental characteristics and by comparison with young of known age. Date of clutch initiation was recorded as observation of the first egg or estimated by back-dating from hatching dates (Robertson et al., 1992).

The date of fledging was not always known and to account for this an expected duration of chick-rearing was used in our statistical analysis (20 days for both species based on Pinkowski et al., 1975; Robertson et al. 1992) and each nest was checked to determine if the chicks fledged prior to or after this tentative post-hatching date. If the fledging date was known then it was used in the analysis. If unfledged chicks were present in the nest after the tentative date then the last fledging date was assumed to be one day after the last observation of unfledged chicks in the nest.

Tree swallows produce a single clutch of eggs per year in Ontario (Robertson et al. 1992). Tree swallows nesting later in the season can be indicative of poorer quality or younger parents with lower reproductive success (Peck and James 1987a; Robertson et al. 1992). Hence we

restricted our statistical sample of nests to those initiated during typical nesting phenology period for tree swallows in Ontario which is prior to 1st June in each year (Peck and James 1987a). Eastern bluebirds can produce one to three clutches per year at this latitude although typically only two clutches are laid usually one during April to May and another after June 1st (Peck and James 1987b). Therefore, we analysed reproductive endpoints of eastern bluebird nests initiated prior to 1st June separately from those initiated after 1st June.

For each species, we tested for reproductive effects of pesticide exposure in tree swallows and eastern bluebirds using the results of all nests monitored during 1988-1994 and, where sample sizes of nests were adequate for analyses, in each year of 1988-1994.

Associations between organochlorine residues and reproduction

To evaluate the relationships between organochlorine residues in eggs and reproduction, we collected eggs (Table 2) and analysed them for 19 pesticides and for polychlorinated biphenyls (see details of chemical analysis for exact compounds). Eggs were usually collected in a single year and were pooled by species and by site and egg contents were analysed as a single pooled sample (Table 2). For tree swallows, one or two fresh eggs per nest were sampled from one to ten nests among sites. For eastern bluebirds, one or two infertile or one fresh egg per nest was sampled from one to five nests per site. Since the half-life of organochlorine pesticides is often over a decade (Edwards 1966) and chlorinated hydrocarbon concentrations in passerine eggs are representative of concentrations in the breeding habitat soils and sediments (Bishop et al 1991; Elliott et al 1994; Johnson et al 1976), we assumed that exposures varied little between years of our study.

To remove any confounding effect of in-use pesticides when we evaluated the relationships between reproduction and organochlorine residues, we only used the reproductive results of tree swallow and of eastern bluebird nests which had not been exposed to in-use pesticides during 1988-1994. For both species, the only nests included in the analysis were initiated prior to June 1st in each year. Since eggs were collected in more than one year at a few

sites, the average of the pooled concentrations was used in the analysis.

Five measures of reproduction were used in the statistical analysis. They were (a) clutch size (b) number of infertile eggs plus number of eggs with confirmed embryonic development which did not hatch ie. eggs infertile plus eggs with early embryonic death (c) number of eggs which failed to hatch which includes all eggs in (b) plus eggs that were lost from the nest without explanation of their fate and eggs not examined for embryonic status ie. non-hatched eggs (d) chicks that died and (e) 'non-fledged eggs' which was the sum of eggs that failed to hatch, chick mortality, and eggs or chicks that went missing without explanation. To determine these parameters, the number of eggs or chicks were summed for each study site. The average clutch size and the proportion of eggs or chicks in categories (b)- (e) were calculated where the divisor for the proportions was the total number of eggs.

A randomization test ($p \leq 0.05$ with sequential Bonferroni test on p values (Rice, 1989)) for trends was used to examine the effect of OC concentration against reproductive success. This test was applied to reproduction data for all years combined (1988-1994) and for each year in which there were at least six study sites. For clutch size a one-tailed test for a declining trend with respect to OC level was performed while a one-sided test for an increasing trend was applied to egg fates in categories (b)-(e).

For each species, we also determined if there was a Spearman correlation (Sokal and Rolhf, 1981) between total organochlorine concentration in eggs and the cumulative toxicity scores per nest calculated for currently-used pesticides (see below). In this analysis toxicity scores were only used for nests initiated before 1st June in each year from study sites where organochlorine concentrations were measured in eggs.

Organochlorine residue analysis

Methods for measuring organochlorine pesticides except endosulfan (see below), chlorobenzenes and total PCB congeners (PCBs) are based on those of Peakall et al. (1986) and Bishop et al. (1996). The pesticides measured were: 1245- and 1234- tetrachlorobenzene,

pentachlorobenzene, α-, β-, and γ- hexachlorocyclohexane, heptachlor epoxide, oxychlordane, trans- and cis-chlordane, dieldrin, cis- nonachlor, photomirex, mirex, pp'DDE, pp'DDD, pp'DDT, op'DDE, op'DDD, and op'DDT. Total PCBs were estimated by determining the sum concentration of 39 PCB congeners measured. These included PCB congeners: #28, 31, 42, 44, 49, 60, 64, 66/95, 70, 74, 87, 97, 99, 101, 105, 110, 118, 129, 137, 138, 141, 146, 149, 151, 153, 158, 170/190, 172, 174, 180, 182/187, 183, 185, 194, 195, 200, 201, 203, 206 (IUPAC number; Ballschmiter and Zell, 1980). Organochlorine residues and PCBs are expressed as µg/g wet weight of body tissue. Detection limits for wet weight OC pesticides, chlorobenzene and PCB residues were 0.0001 ug/g wet weight.

Endosulfan

The samples were blended with acetonitrile: water and then partitioned with methylene chloride. A Florisil cleanup of extracts was performed prior to quantitation on Hewlett Packard 5890 Series II gas chromotograph equipped with an electron capture detector. Recoveries of standards were 85-100%. Detection limit for endosulfan was 0.002 ug/g wet weight.

Associations between toxicity and frequency of spray events during 1988-1994

Pesticides used in orchards during the study are not bioaccumulative in bird eggs so we lacked actual concentrations of exposure for evaluating the relationship between exposure and reproductive response. Therefore, we calculated each nest's chemical exposure based on spray application rate, extent of the farm sprayed and the relative avian toxicity of each chemical (Appendix 1). The experimental unit of exposure was the nest since we assumed fates of all offspring would be correlated.

The toxicity score for each nest was calculated as:

$$(\text{FACTOR})(\text{RATE}) / (\text{INDEX})$$

FACTOR was the surface area of the farm sprayed. This was described on the spray schedules and ranged from 100% of the orchard to approximately 75%; 50%; 33%; 25%;12.5% of the orchard sprayed; 5% for border spray of orchard or base of trees only and 1% if sprays

were only on trees in the row bordering the farm home. RATE was the application rate of each chemical converted to kilograms of active ingredient/ hectare. INDEX was a toxicity index for each chemical. Acute toxicity indices were derived from the amount of the chemical necessary to reach the LD 50 for a model adult tree swallow (based on typical weight of 20 g; Robertson et al., 1992) or an adult eastern bluebird (35 g; Bishop, unpublished data) based on literature values for LD 50s for a variety of avian species (Table 1; see also Appendix 2). To determine the amount of chemical necessary to reach the LD50 for each chemical we used a the value of 27.9 ug/g based on a 1 kg active ingredient /ha as the concentration of pesticide residue expected to occur on insects eaten by insectivorous birds in sprayed agricultural areas (Appendix 2: Table 2). This was the mean concentration of several measurements reported for insects collected after spray events or extracted from animals poisoned by organophosphorus insecticides (Table 2). Despite the variation in chemical types, there are no comparable literature values for other types of pesticides therefore we used 27.9 ug/g as generally representative of all pesticides. The chronic toxicity index was based on the amount of chemical required to reach the allowable daily intake (ADI) for the model tree swallow or model bluebird (Appendix 2). Since ADIs have not been developed for birds for all the compounds used in the orchards, the ADIs used were those developed for humans (International Programme on Chemical Safety, 1996).

The daily exposure for each nest was calculated as the summed toxicity scores of all chemicals sprayed per day. The cumulative exposure for each nest was calculated as the sum of all the daily exposures up to and including the last day of exposure being examined in the statistical analysis. The differences in cumulative toxicity scores among species, nesting periods and years were determined with Mann-Whitney tests or Kruskal-Wallis tests using a Tukey's test for unequal sample sizes (Sokal and Rohlf, 1981).

For the analysis of assocations between toxicity score and reproduction, we designated three phases of reproduction: pre-incubation, incubation and chick-rearing. Since parent birds of both species spend time nest building prior to clutch initiation (Pinkowski et al., 1975; Robertson

et al. 1992), they may be exposed to sprays at that time and we designated that as the pre-incubation period. We designated this period as beginning seven days prior to the date of clutch completion and this period varied because clutches varied in size. Incubation continued from the day of clutch completion until the date of that the last egg hatched (Pinkowski et al., 1975; Robertson et al. 1992). Chick-rearing continued from the hatching date until all chicks fledged or were found dead in the nest.

Statistical analysis of reproductive data for each species was examined for clutch size, egg fertility, and daily survival rates of eggs and chicks (Appendix 1). A weighted linear regression was used to examine the relationship between average clutch size and average daily toxicity scores during the pre-incubation period using the number of nests as a weighting factor (Sokal and Rohlf, 1981). The analysis of the relationship between daily toxicity score and proportion of fertile eggs was a logistic regression based on the logarithmic transformation of the total pre-incubation exposure including a term for the background proportion of fertile eggs. The weighted regressions were run using log-transformed proportions of either clutch size or proportion of viable eggs.

Survival probabilities for eggs (Mayfield 1961; 1975) relative to the cumulative toxicity scores during the pre-incubation period and survival probabilities for chicks relative to cumulative toxicity scores during the pre-incubation through the chick-rearing period were assessed by a maximum likelihood estimation significance test. The curve fitting was done using a logarithmic transformation of the toxicity score. Daily survival rates were fitted using logistic regression including a term for background survival rates for each of the eggs and chicks separately. The significance of the effect of exposure was assessed by calculating the difference in the predicted probability of survival from a non-exposed nest and a nest which received the maximum observed exposure. The frequency of times the randomized difference was as extreme as the observed difference was used as the measure of significance after a sequential Bonferroni analysis of the calculated p values was performed. The sequential Bonferroni analysis

was based on p=0.05 applied to the number of correlations performed on results for each year, species and time period of nesting. The difference was used because the estimates of the slope and ED 50 couldn't be used to order the magnitude of the effect of exposure. We ran 1000 randomizations including the observed data as one randomization (Appendix 1; Sokal and Rohlf, 1981). The analyses were performed using SAS for PC.

RESULTS

Organochlorine residues in eggs

Eastern bluebird eggs were often more contaminated with organochlorine pesticides than tree swallow eggs from the same locations (Table 3). In both species, concentrations of all organochlorine pesticides were low (<0.23 wet wt. ug/g) except pp'DDE which was detected at much higher levels and showed high variability among sites (Table 3). Summed PCB concentrations were also low in eggs of both species. Among sites, PCB concentrations ranged from 0.27 to 0.71 ug/g wet weight (w.w.) in tree swallow eggs. For eastern bluebird eggs, the range for total PCB concentration among sites was 0.07 to 0.87 ug/g (w.w.).

The concentration of pp'DDE dominated the contaminant burden in the eggs. However, to account for the possible effects of other chemicals or interactions between compounds we analysed the relationships among reproductive endpoints and organochlorine concentrations in eggs based on the summed concentration of all compounds found in eggs of each species.

Associations between organochlorine residues and reproduction

For tree swallows, there were 10 sites with a total of 200 nests that were not sprayed in 1988-1994. Only one year, 1993, had at least six sites which had nests that were not sprayed (N=66 nests). For 1988-1994 and 1993 alone, there were no significant trends between increased total OC concentration in eggs and reproductive endpoints (Table 4).

For eastern bluebirds, there were 12 sites with nests that were not sprayed during 1988-1994 (N=67 nests). The only year with sufficient sample sizes of sites and nests for analysis was

1992 (six sites; N=19 nests). For the combined sample of nests not sprayed during 1988-1994, there were significant associations between increased organochlorine concentrations in eggs and increased occurrence of unhatched eggs(Table 4; Fig.1). For 1992, there were no significant trends, probably due to the small sample size of nests without a strong gradient in organochlorine concentrations among sites (Table 4).

Toxicity Indices and Toxicity Scores

The compounds with the lowest toxicity indices and hence the highest toxicity to birds were the organophosphorus and carbamate insecticides. Six of the ten compounds with acute toxicity indices less than 30 mg were OP and CARB compounds. Other compounds with low toxicity indices were sulfur, and oil and the organochlorine insecticide endosulfan (Table 1). Sulfur was only applied in one orchard. It is used as a substitute for fungicide pesticides. Oil was always applied as a scale insect pesticide in early spring, usually in April, and occasionally applied in May or June. Therefore early nests rather than nests initiated after 1st June would be more often exposed to oil. Compounds with acute toxicity indices greater than 30 mg to over 100 g were the synthetic pyrethroids, ethylene-bis-dithiocarbamate and ergosterol synthesis inhibiting fungicides, bordeaux mixtures, and nutrients and minerals (Table 1).

Acute and chronic toxicity indices (Table 1) were highly correlated (Spearman R=0.56; p=0.00008). Therefore acute toxicity indices were used in the calculations since more species had been tested and the methods and results of acute toxicity studies were more comparable than chronic reproductive studies.

The toxicity scores for nests based on toxicity indices, rate of application and extent of the farm sprayed (Table 5) were similar in most years for tree swallows and eastern bluebird nests initiated before 1st June (early nests) although in 1989-1991 mean and maximum toxicity scores for tree swallow nests were slightly lower than in bluebirds (Table 5). The bluebird nests initiated after 1st June (late nests) had much higher inter-year variation in toxicity scores than the early nesters. The maximum toxicity scores were very high in 1989 and 1994 whereas the mean and

maximum scores were lower than average compared to other years in 1990, 1991 and 1993.

Associations between organochlorines in eggs and cumulative toxicity scores

During 1988-1994, there were 388 tree swallow nests initiated prior to 1st June on study sites where organochlorine chemicals were measured in eggs. The cumulative toxicity score for those nests for the pre-incubation through to fledging period varied from 0 to 3.276 while total organochlorine concentrations varied from 0.74 to 3.5 ug/g. These variables were significantly and positively correlated (Spearman rank R= 0.43; p=0.001; Fig.2).

On farms where organochlorines were measured in eastern bluebird eggs, there were 166 nests initiated prior to 1st June. Cumulative toxicity scores varied from 0 to 4.147 while organochlorine concentrations varied from 0.47 to 106.3 ug/g (Spearman rank R= 0.34; p=0.001). This correlation appears to be the result of the large number of observations below 0.10 for cumulative toxicity score and less than 2.0 ug/g organochlorine concentration (Fig. 3) and hence may be spurious.

Associations between Toxicity Scores and Reproduction

Tree swallow

There were 455 nests used in the analysis of 1988-1994. Among years, sample sizes varied between 18 and 144 nests (Table 6). The total number of eggs evaluated was 2598 (Table 5).

There was no evidence of declining clutch size with increased daily toxicity scores per nest during pre-incubation (Table 6). For four of seven study years egg fertility declined significantly with increased cumulative toxicity score during pre-incubation (Table 6). For all study years combined and in 1991 there was a marginal decline (p=0.022-0.049) in egg fertility (Table 6). Similarly, daily egg survival declined as cumulative toxicity score per nest increased during incubation in all years except 1988 and 1990 and the trend during 1988-1994 showed a marginal but not significant decline (Table 6; Fig. 4). Daily chick survival also declined significantly with

increased cumulative toxicity scores during chick-rearing in three of seven years with marginal declines in two other years (Table 6; Fig.4). The magnitudes of these declines were often small (see Decline (b) Table 6) but for the two daily survival probabilities the cumulative effect over the incubation or chick-rearing periods could be substantial. In some years the decline in fertility was as high as 13%. Impacts on egg and chick survival, though statistically significant, were often less than 1% while in one year they were as high as 14% (Table 7).

Eastern Bluebird

There was a total of 385 nests used in the analysis of 1988-1994 reproductive data. Within years sample sizes varied between 5 and 44 nests (Table 5). Statistical analysis was not performed on the results of 1988 when only 5 to 7 nests were sampled although those results were used in the analysis of the combined data set. The total number of eggs whose fate was evaluated was 930 for early nesters that initiated nests prior to 1st June and 796 eggs for nests initiated after 1st June (Table 5).

There was no evidence that clutch size declined with increased daily toxicity scores during pre-incubation (Table 7;8). In early nests, there was one year that the proportion of fertile eggs declined significantly with increased cumulative toxicity scores and overall 1988-1994 there was a marginally significant trend ($p=0.026$) of this type (Table 7). In late nests there was only one year showing a significant decline in egg fertility (Table 8).

The daily survival rate of eggs and daily survival rate of chicks in early nests were not significantly associated with current spray exposure (Table 7). However, there were several years with significant trends in the late nesters. Daily egg and daily chick survival decreased significantly with increased toxicity score in three years (Table 8). These declines in survival were significant but not often high. The maximum decline in survival was only 4% (Table 8).

DISCUSSION

Both tree swallows and eastern bluebirds experienced significant declines in reproduction

with increased pesticide exposure and toxicity. In particular, egg survival was affected. Tree swallows appeared to be more sensitive than eastern bluebirds to the effects of pesticides applied during 1988-1994. In tree swallows, declines in egg fertility and daily egg and chick survival were associated with increased toxicity scores of pesticides in several years. The early nesting bluebirds were exposed to about the same range of pesticide toxicity scores as tree swallows but showed few declines in fertility or survival. In contrast, later nesting bluebirds were exposed to different patterns and degrees of toxicity than the early nesting birds. They experienced significant decreases in egg fertility, egg survival and chick survival particularly in 1994 when toxicity scores for some nests were extremely high. However, it also appears that the high organochlorine concentrations in bluebird eggs in some orchards are as toxic as pesticides currently in use.

Similar effects have been reported in birds exposed to organochlorine pesticides and OP and CARB pesticides in the wild and in controlled laboratory experiments. Reduced egg viability and weight, hatching success, and chick survival are found in captive bengalese finches (*Lonchura striata*) and ringed doves (*Streptopelia risoria*) dosed with pp'DDT and/or pp'DDE (Jefferies 1967; 1971; Keith and Mitchell 1993). Concentrations accumulated in bengalese finch eggs were 10- 100 times higher than in most eastern bluebirds eggs in our study although one of the study sites had concentrations in eggs that were comparable to bengalese finches. American robins (*Turdus americanus*) nesting in orchards during the 1960s and exposed to intensive DDT use as well as OP, carbamate insecticides, lead arsenate and fungicides showed an increase in occurrence of one egg clutches but a decrease in four egg clutches and significantly lower mean clutch size than in reference sites (Johnson et al 1976). Tree swallows nesting in orchards in the Okanagan Valley were contaminated with up to 11 ug/g pp'DDE and experiencing exposure to in-use pesticides during the 1990s had lower mean clutch size than those from non-orchard sites but the differences were not significant and hatching or fledging successes did not show any differences among exposure areas (Elliott et al 1994). Although none of these studies on

songbirds reported any indication of eggshell thinning, embryotoxic effects in passerines are consistent with findings in other avian groups such as the pelicaniformes and raptors which show both embryotoxicity and eggshell thinning in response to OCs in eggs (Newton et al 1986).

Fleutsch and Sparling (1994) found significantly reduced hatching success in American robins and mourning doves sprayed orchards as compared to organic apple orchards in one of two years of their study. Fledging success was also affected although significant effects were only found in robins in one year of the study with marginally significant effects in mourning doves in the other year of the study. Daily survival rates of nests of both species were significantly lower in one study year but only marginally lower in the other year.

Patnode and White (1991) pooled their reproduction results in pecan orchards for three species, northern mockingbird (*Mimus polyglottus*), northern cardinal (*Cardinalis cardinalis*), and brown thrasher (*Toxostoma rufum*), and found that daily egg and chick survival rates varied significantly as increased in calculated pesticide toxicity scores increased. This score was similar to ours in that it was determined as the product of the acute toxicity of the compound sprayed and the number of times it was sprayed with a divisor of the number of days that the nest was active. We refined this approach by accounting for the extent of the farm sprayed and the application rate as well as determining the cumulative exposures during development of eggs and chicks for specific periods of development. Their results were also similar to ours in that they found daily egg survival rates were significantly lower in the two highest classes of toxicity scores in one study year and in both years chick survival was also reduced significantly in the nests with the highest toxicity scores. Patnode and White (1991) reported that the most toxic compounds used in pecan orchards were the OP and CARB pesticides. Like Fleutsch and Sparling (1994), they attributed effects to OP and CARB use in the orchards although they did not measure OCs in eggs. .

As in this study, earlier investigations found inter-year differences in the statistical significance of their findings however the downward trend in egg fertility, and egg and chick

survival with increased exposure to more toxic compounds consistently occurred although it was not always significant. Fleutsch and Sparling suggest that differences in reproductive effects between years was likely due to spraying of a greater variety of insecticides with high acute toxicity including azinphos-methyl, dimethoate and methomyl in one of two years of the study. Patnode and White suggest that differences in reproduction between years was due to a more severe effect and persistence of pesticides during a drought in 1988 as compared to normal rain and cooler temperature patterns in the second year of the study. We did not monitor weather conditions but our analysis of the combined results of all years should reduce the confounding effects of weather among years. We found significant associations between pesticide toxicity scores and reduced reproductive success in nests in certain years but not all years suggesting weather or other factors play a role in determining reproductive success within these orchards from year to year.

The results of a behavioural study conducted in 1996 in one of the orchards monitored during 1988-1994 indicate effects of chemicals in the orchards are likely due to a direct effect on the developing embryo or chick. The use of fungicides and OP insecticides had no effect on incubation periods maintained by tree swallow females (Bishop et al, unpublished) which suggests that reduced tree swallow egg viability is more likely associated with direct pesticide exposure on the eggs. Possible factors might be direct pesticide contact with the egg via feathers or brood patch of the parent bird or subtle changes induced by OP exposure on the parents' ability to thermoregulate itself (Rattner et al., 1982) and possibly its eggs. In the same behavioural study, there were no effects of insecticide use on tree swallow chick growth (Bishop et al, unpublished). This also points to mechanisms by which chicks may be affected by spray exposures in orchards. It suggests direct pesticide exposure may be reducing tree swallow chick survival through contact with pesticides rather than lack of food resources. Although this is consistent with marginal cholinesterase depression in tree swallow nestlings and significant cholinesterase depression in bluebird chicks in these orchards reported by Burgess et al (1998),

food reduction by insecticides may be a factor at other locations outside of the sites studied here and should not be discounted.

Taken together, studies indicate that birds nesting in orchards are lethally and sublethally affected at every critical stage of reproduction and development by pesticides used in orchards during the 1990s. In total, there are now six species of songbirds reported to experience significantly reduced reproductive success associated with the use of pesticides in orchards (Patnode and White, 1991; Fleutsch and Sparling 1994). While most of these species have not demonstrated any overall downward population trends in North America, population sizes of eastern bluebirds have been of concern in the recent past. The eastern bluebird has been considered a species at risk in Canada (Risley, 1984) although populations have increased substantially and current recommendations (Read and Alvo, 1995) are that the species be downgraded from a listed species in Canada. This recovery is generally attributed to the popularity of bluebird nest box trails and mild winters in recent years (Read and Alvo, 1995). Many nest boxes are located in rural areas and occasionally within or on the edge of sprayed orchards (McNicholl et al, 1994) therefore the locations of the boxes and potential effects on their eggs should be of concern to trail operators.

The chemicals implicated in reduction of reproductive rates in passerines in other studies in orchards have often been organophosphorus and carbamate insecticides. Our study also indicates that these compounds are potentially highly toxic to birds although we also showed that the organochlorine compounds, in particular DDE, are likely to affect egg survival at concentrations found in some orchards. Other compounds such as oil and sulfur appear to be potentially toxic when birds are exposed. Although the fungicides used in these apple orchards appear to have low toxicity to birds, they are applied repeatedly at high rates and often in combination with other chemicals and do contribute to the overall toxicity scores of nests exposed in this study. Since there were no nests that were exposed to OP and CARB alone it is difficult to discern whether their impact is significant alone or in combination with other

compounds and this needs to be the focus of future studies.

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1.1) STATISTICAL MODEL

The reproductive success is described in terms of 4 parameters: i) clutch size, ii) proportion of fertile eggs, iii) the egg daily survival rate during incubation and iv) the chick daily survival rate during chick rearing. Each of these parameters could be affected by exposure to spraying. Define

n(x) - number of eggs in clutch at exposure x,
$P_F(x)$ - proportion of fertile eggs at exposure x,
$P_I(x)$ - daily survival rate for fertile eggs during incubation at exposure x and
$P_R(x)$ - daily survival rate for chicks during chick rearing at exposure x.

Exposure to a chemical could affect the clutch size or any of the three survival probabilities. The clutch size and survival probabilities must be modeled differently because of the nature of the variables i.e. the count is restricted to be non-negative while the probabilities are constrained to fall between 0 and 1.

The average clutch size is assumed to be linearly related to the pre-incubation exposure viz.

$$E(n(x)) = \alpha_C + \beta_C x$$

while the survival probabilities will be assumed to be linearly related to exposure after a logit transformation with a background survival level. The proportion of fertile eggs is modeled as

$$P_F(x) = \alpha_F \frac{\exp(\beta_F + \gamma_F x)}{1 + \exp(\beta_F + \gamma_F x)}$$

where α_F denotes the background proportion of viable eggs when there is no exposure, β_F denotes the intercept term for the logistic regression and γ_F denotes the slope of the logistic regression and x denotes the log of the exposure. The models for the daily survival probabilities for incubation and chick rearing are defined similarly.

1.2) LIKELIHOOD EQUATION

The analysis of the clutch size is done using a linear regression and doesn't require use of a likelihood equation. The analysis of the survival probabilities, however, is done through calculating maximum likelihood estimators of the probabilities. There are 6 different egg fates which are recorded in the data file (Table 1). The likelihood equation must be written separately for each of the egg fates and the resulting terms multiplied together to give the overall likelihood equation. The contribution to the likelihood equation for each egg fate is given in Table 2.

1.3) ASSESSING SIGNIFICANCE

The experimental unit for this type of study is the nest since the fates of all offspring in the same nest are correlated. Nest abandonment or death of a parent causes the death of all offspring. Alternatively death of one nestling could improve the survivability of it's nest mates by reducing parental burden.

If the exposure has no effect on reproductive success then the reproductive success of a nest would be unchanged even under a different exposure scenario. However, the calculated trend in reproductive success with dose would not be identically zero because of the random nature of observations. The distribution of the calculated trend under the assumption of no exposure effect can be simulated by randomly reallocating the exposure scenarios among the nests and calculating the trend. The significance of the observed effect of exposure can be assessed by comparing observed trend with this simulated distribution of trends.

The exposure dates for each nest were converted to days prior to or after the last hatching date. The resulting exposure could be associated with any nest after random reallocation of exposure among nests. This procedure will sometimes produce exposure days which are inappropriate for a given nest. E. g. an exposure for the first day of susceptibility for a large nest might occur 28 days prior to last hatching and if this exposure were randomly associated with a small nest then it might occur before the first susceptible date for the small

nest. In these instances the exposures prior to the first susceptibility date were discarded and similarly exposures after the last egg or young was seen were discarded.

The significance of the effect of exposure on the survival probabilities was assessed by calculating the difference in the predicted probability of survival from a non-exposed nest and a nest which received the maximum observed exposure. The frequency of times the randomized difference was as extreme as the observed difference was used as the measure of significance. The difference was used because the effect of the exposure on the survival probabilities was small over the range of observed exposures. Because of this the estimates of slope and ED50 are unstable and not suitable for assessing significance.

2) ANALYSIS PROGRAMS

The data analysis is done through a series of 3 programs. The first program massages the nest information to combine the nest visit and nest summary files. The second program reads the spray schedule data and relates this information to the massaged nest file to extract the exposure information for each nest and the last program does the curve fitting and significance testing. These three programs could be combined but it was more convenient to write and debug them separately.

2.1) NEST MASSAGE PROGRAM:

This program combines the nest summary and nest visit information onto one file and produces a code for the fate of each egg in the clutch. A line in the output file gives dates of clutch initiation, clutch completion, first and last hatching date and the first and last fledging date. Each egg in the clutch is coded as having one of 6 fates as described in Table 1. In addition the last day each offspring known to be alive/present and the first date it is known to be dead/missing are stored.

In general the date of fledging is unknown and it is only recorded how many fledglings were in the nest on certain dates. Sometimes the dates recorded in the file were months after the

last hatching date and clearly don't reflect the date at which the fledging occurred. The data analysis, however, requires a fledging date in order to determine when the nestlings stop being exposed to chemicals and in order to calculate the number of days at risk when calculating the chick rearing survival probability. The duration of chick rearing is an input parameter to the program. The value is added to the last hatching date to provide a tentative value for the fledging date. If chicks fledge prior to this tentative date then the reported value is used as the fledging date. If unfledged chicks are present in the nest after the tentative date then the last fledging date is assumed to be one day after the last observation of unfledged chicks in the nest.

2.2) SPRAY EXPOSURE PROGRAM

This program reads the output of the nest massage program, the spray schedule data and the toxicity information and prepares a file giving the date and toxicity score for each exposure for the nest. The log of the spray exposures is stored in the output file since the toxicity scores vary over several orders of magnitude. The number of susceptible days prior to clutch initiation is a input parameter to the program.

The program stores the exposure information from the first date of susceptibility until the last day of fledging. In cases where none of the young fledge the projected date of fledging based on the hatching date is used.

2.3) MODEL FITTING PROGRAM

2.3.1) EXPOSURE RESPONSE TABLES

The analysis begins by calculating an exposure response table which shows the mean response at each exposure level. The mean clutch size and the proportion of fertile eggs are tabulated for the daily average and total pre-incubation exposure respectively. The proportion of incubation days survived over days at risk is tabulated for incubation exposure and the proportion of chick rearing days survived over days at risk is tabulated for chick rearing exposure. Incubation exposure and chick rearing exposure were defined as the cumulative exposure from the first susceptible day.

Define N_{Ai} as the number of eggs of fate A in nest i and similarly define N_{Bi} , N_{Ci} , N_{Di} , N_{Ei} and N_{Fi} . The total of all eggs of fate A is defined as N_A and the total for other fates is defined analogously.

The exact fate of eggs/clicks coded to fates C or E is undetermined. In order to prepare the exposure-response table these eggs were apportioned among infertile, egg failure and chick death in proportion to the probabilities recorded for eggs with fates A, C and D. The eggs with fate code C were apportioned as $f_A = N_A / (N_A + N_B)$ to infertile and $(1- f_A)$ to egg mortality. Similarly eggs with fate code E were apportioned as $g_A = N_A / (N_A + N_B + N_D)$ to infertile, $g_B = N_B / (N_A + N_B + N_D)$ to egg mortality and $(1- g_A - g_B)$ to chick mortality. Hence, for the fertility exposure-response table response was a fraction with clutch size N_i as the denominator and $N_i - N_{Ai} - f_A N_{Ci} - g_A N_{Ei}$ as the numerator.

Since the nests are not visited daily the exact day on which an egg/young fails isn't known and exposure rates varied daily. A daily exposure response for each egg was defined as follows. Let D_1 denote the last day the egg/chick was alive/present and D_2 denote the first day it was known to be dead/absent. For each day up to and including D_1 the egg contributed a value of 1 to both days at risk and days survived. For any day k $(D_1 < k \leq D_2)$, days at risk was defined as $D_2 - k + 1$ and days survived as $D_2 - k$. The equation used to tabulate the exposure response table for each egg are shown in Table 3.

These tables provide the basis for the analysis of clutch size and initial estimates of the effect of exposure on survival probabilities. The tables also provide the basis for the graphs.

2.3.2) ESTIMATING RESPONSE TRENDS

A weighted regression was run relating the average clutch size to the pre-incubation exposure using the number of nests as the weighting factor. The three survival probability variables were transformed using a modified logit:

$y = \log(p/(1-p))$

where

$p = 1/4n$ if $x=0$
$= x/n$ if $0<x<n$
$= 1 - 1/4n$ if $x=n$

x = number surviving and
n = number at risk

A weighted regression was run relating the transformed proportions to the exposure using np(1-p) as the weighting term. The resulting estimates of intercept and slope were used as initial values for a numerical maximization of the likelihood equation. The numerical maximization was done using the Nelder-Mead simplex method.

The program also provides the option of inputting an initial value for the numerical maximization. By starting the curve fitting from different points in the parameter space the stability of the convergence of the Nelder-Mead algorithm can be assessed.

2.3.3) ASSESSING SIGNIFICANCE

The randomization procedure is done through randomly assigning an observed spray schedule to each nest. The assignment is done without replacement. So that each spray schedule is assigned to one nest during each randomization trial. The curve fitting is then redone starting at the final value for the maximum likelihood estimate for the actual data.

The randomization procedure is time consuming. For the analysis of the tree swallow data for all years performing 100 randomizations took approximately 1 hour of computing time. The Nelder-Mead algorithm assumes the curve fitting has converged when the variability among the fitted values at the vertices of the simplex of test values is small.

The significance was calculated including the observed data as one randomization. This ensures the estimated probability can't equal zero.

Table 1: Offspring fate codes

Fate Code	Description
A	non-fertile egg
B	fertile egg which fails to hatch
C	egg which fails to hatch but has unknown viability (includes missing eggs and eggs whose development status wasn't assessed)
D	hatched chick which fails to fledge
E	egg/chick which is missing and hatching status in unknown (may have been a non-fertile egg)
F	fledged young

Table 2: Likelihood equations for each offspring fate code

Fate Code	Likelihood
A	$1 - P_F(x_F)$
B	$P_F(x_F) \prod_{i=L}^{D_1} P_I(x_i) \left[1 - \prod_{i=D_1+1}^{D_2} P_I(x_i)\right]$
C	$1 - P_F(x_F) \prod_{i=L}^{D_2} P_I(x_i)$
D	$P_F(x_F) \prod_{i=L}^{H} P_I(x_i) \prod_{i=H+1}^{D_1} P_R(x_i) \left[1 - \prod_{i=D_1+1}^{D_2} P_R(x_i)\right]$
E	$1 - P_F(x_F) \prod_{i=L}^{H} P_I(x_i) \prod_{i=H+1}^{D_2} P_R(x_i)$
F	$P_F(x_F) \prod_{i=L}^{H} P_I(x_i) \prod_{i=H+1}^{F} P_R(x_i)$

$P_F(x)$ - probability an egg is fertile given exposure x
$P_I(x)$ - daily survival probability during incubation given exposure x
$P_R(x)$ - daily survival probability during chick rearing given exposure x

x_i - exposure on day i

L - day of nest completion
H - day of last hatching
F - day of fledging
D_1 - last day egg/young known to be alive/present
D_2 - first day egg/young known to be dead/absent

Table 3: Equations used in calculating exposure- response tables

Egg Daily Survival Rate

Fate	Day	Days at Risk	Days Survived
B	$k \le D_1$	1	1
	$D_1 < k \le D_2$	1	$(D_2 - k) / (D_2 - k + 1)$
C	$k \le D_2$	$1-f_A$	$(1-f_A)(D_2 - k) / (D_2 - k + 1)$
D	$k \le H$	1	1
E	$k \le D_2$	g_B	$g_B (D_2 - k) / (D_2 - k + 1)$
	$k \le H$	$1-g_A-g_B$	$(1-g_A-g_B)(D_2 - k) / (D_2 - k + 1)$
F	$k \le H$	1	1

Chick Daily Survival Rate

Fate	Day	Days at Risk	Days Survived
D	$k \le D_1$	1	1
	$D_1 < k \le D_2$	1	$(D_2 - k) / (D_2 - k + 1)$
E	$H < k \le D_2$	$1-g_A-g_B$	$(1-g_A-g_B)(D_2 - k) / (D_2 - k + 1)$
F	$H < k \le F$	1	1

where
H - day of last hatching
F - day of fledging
D_1 - last day egg/young known to be alive/present
D_2 - first day egg/young known to be dead/absent
$f_A = N_A / (N_A + N_B)$
$g_A = N_A / (N_A + N_B + N_D)$
$g_B = N_B / (N_A + N_B + N_D)$

Appendix 2. Formulas for acute and chronic indices for pesticides

Acute Index (mg)

1. LD50 (mg/kg) is converted by arithmetic to the amount (mg) required to reach LD50 for a 20 g bird. This value is **X** mg of the chemical.

2. Concentration of the chemical residue (ug/g) on insect food has been calculated as 27.9 ug/g (see Chapter four text; Table 2) for every 1 kg/ha of active ingredient applied. Using the known application rate of active ingredient for each chemical (kg/ha) (see Chapter four; Table 1) the residue of chemical (mg) expected in 1 kg (ie. 1×10^6 mg) of insect food can be calculated.

3. The Acute Index (mg) is the amount of insects the 20g bird must eat to reach the LD50. Calculated as:

Amount of insects (mg) to be consumed to reach LD50

= (**X** mg of chemical) (1×10^6 mg insect food) / Chemical residue in 1×10^6 mg insect food

Chronic Index (mg)

1. The allowable daily intake per day (ADI) is expressed as mg/kg chemical /day. This is converted to the amount of chemical (mg) that a 20 g bird can consume per day by simple arithmetic. This value is **X** mg of the chemical.

2. Concentration of the chemical residue (ug/g) on insect food has been calcluated as 27.9 ug/g (see Chapter four text; Table 2) for every 1 kg/ha of active ingredient applied. Using the known application rate of active ingredient for each chemical (kg/ha) (see Chapter four; Table 1) the residue of chemical (mg) expected in 1 kg (ie. 1×10^6 mg) of insect food can be calculated.

3. The Chronic Index (mg) is the amount of insects the 20g bird must eat to reach the ADI. Calculated as:

Amount of insects (mg) to be consumed to reach ADI

= (**X** mg of chemical) (1×10^6 mg insect food) / Chemical residue in 1×10^6 mg insect food

Table 1. Application rates, acceptable daily intakes, LD50s, and chronic and acute toxicity indices for pesticides and other substances sprayed on apple orchards (1988-94)

No. Times Sprayed	Technical Name or other names	Chemical Name	Chemical Type
388	Dithane	mancozeb	EBDC
353	Polyram	metiram	EBDC
230	Imidan	phosmet	OP
220	Stop It	calcium	MIN
206	Guthion, APM	azinphosmethyl	OP
151	Manzate	maneb	EBDC
149	Captan 50%	captan	Phthalate
129	Nova	myclobutanil	EBI
88	Superior Oil	oil	Hydrocarbon
81	ZoloneFlo	phosalone	OP
66	Omite 30 W	propargite	Metal/Mineral/Sulfur
58	Ripcord 400 EC	cypermethrin	SP
39	Dikar	Dikar	dinocap & mancozeb
35	Diazinon	diazinon	OP
32	Thiodan	endosulfan	OC
32	Kelthane AP 35	dicofol	OC
28	Decis	deltamethrin	SP
25	Sevin 50% WP	carbaryl	CARB
24	Agri-Mycin, Strep	streptomycin	Antibiotic
22	Apollo	clofentezine	Tetrazine
21	Benlate	benomyl	Benzimidazole
19		sulfur	
16	Lannate	methomyl	CARB
11	Carzol 92 SP	formetanate hydrochlor	CARB
10	MgSO4	magnesium	MIN
9	Phygon-XL	dichlone	Quinone
9		epsom	salt
9	Morestan	oxythioquinox	Quinoxaline
9	NAA, Fruitone-N	napthaleneacetic acid	Quinoxaline
8	Cyprex	dodine	dodecylguanidine acetate
5	Plictran	cyhexatin	
5	B-Nine, Alar	daminozide	plant growth regulator
2	Belmark	fenvalerate	SP
2	Supracide	methidathion	OP
1		Bordeaux	Copper sulphate
1	Cygon 480E	dimethoate	OP
1	NAD	naphthaleneacetamide	Quinoxaline
1	Pirimor	pirimicarb	CARB

Table 1(CONTINUED). Application rates, acceptable daily intakes, LD50s, and chronic and acute toxicity indices for pesticides and other substances sprayed on apple orchards (1988-94)

Chemical Name	Application rate (kg or litres active ingredient/ ha)	ADI * (mg/kg/day)
mancozeb	4.8 kg	0.03
metiram	4.8 kg	0.03
phosmet	1.9 kg	0.02
calcium	1.9 kg	1
azinphosmethyl	1.05 kg	0.005
maneb	4.8 kg	0.03
captan	3.0 kg	0.1
myclobutanil	0.14 kg	0.003
oil	60 L	1
phosalone	1.0 kg	0.001
propargite	1.65 kg	0.15
cypermethrin	0.1 L	0.05
Dikar	0.38 kg	0.001
diazinon	1.63 kg	0.002
endosulfan	2.0 kg	0.006
dicofol	1.58 kg	0.002
deltamethrin	0.0125 L	0.01
carbaryl	1.5 kg	0.01
streptomycin	0.45 kg	0.03
clofentezine	0.15 L	0.02
benomyl	0.55 kg	0.02
sulfur	125 L	1
methomyl	1.45 kg	0.03
formetanate hydrochlor	1.01 kg	0.004
magnesium	15.0 kg	1
dichlone	0.42 kg	0.175
epsom	15.0 kg	1
oxythioquinox	0.56 kg	0.006
napthaleneacetic acid	0.035 kg	0.005
dodine	1.46 kg	0.01
cyhexatin	0.66 kg	0.1
daminozide	6.48 kg	0.5
fenvalerate	0.135 L	0.02
methidathion	1.58 kg	0.001
Bordeaux	18.0 kg	0.5
dimethoate	2.04 L	0.01
naphthaleneacetamide	0.126 kg	0.005
pirimicarb	0.85 kg	0.02

Table 1(CONTINUED). Application rates, acceptable daily intakes, LD50s, and chronic and acute toxicity indices for pesticides and other substances sprayed on apple orchards (1988-9

Chemical Name	Median LD 50 (mg/kg)	Reference for LD 50	Chronic Toxicity Index	Acute Toxicity Index
mancozeb	2000	a ; b	3.7	246.91
metiram	2000	a ; b	4.17	277.78
phosmet	1033	a; c ; d ; e	7.02	362.46
calcium	2000	a	133.33	266.67
azinphosmethyl	32	a	3.17	20.32
maneb	2000	a	4.17	277.78
captan	2000	a	22.22	444.44
myclobutanil	510	a	14.29	2428.57
oil	2000	a	11.11	22.22
phosalone	1000	a	0.67	666.67
propargite	2000	a	60.61	808.08
cypermethrin	2000	a	333.33	13333.33
Dikar	2000	a	11.4	-
diazinon	6.2	a ; d ; e	0.82	2.54
endosulfan	73.5	a ; d ; e	2	24.5
dicofol	325	c ; d	0.84	137.13
deltamethrin	2000	a	533.33	106666.67
carbaryl	2000	c ; d ; e	4.44	888.89
streptomycin	2000	a	44.44	2962.96
clofentezine	2000	a	88.89	8888.89
benomyl	100	e	16.5	121.21
sulfur	2000	a	5.33	10.67
methomyl	23	f	13.79	10.57
formetanate hydrochlor	32	a ; f	2.64	21.12
magnesium	2000	a	44.44	88.89
dichlone	2000	a	277.78	3174.6
epsom	2000	a	44.44	88.89
oxythioquinox	2000	a	7.14	233.33
napthaleneacetic acid	2000	a	95.24	38095.24
dodine	2000	a	4.57	913.24
cyhexatin	595	a	101.01	601.01
daminozide	2000	a	51.44	205.76
fenvalerate	2000	g ; h	98.77	9876.54
methidathion	52.5	c ; d ; i	0.42	22.15
Bordeaux	2000	a	37.04	74.07
dimethoate	26	a ; b	3.27	8.5
naphthaleneacetamide	2000	a	26.46	10582.01
pirimicarb	20	a ; c	15.69	15.69

Notations for Table 1:

* ADI = Acceptable Daily Intake

EBDC = ethylene-bis-dithiocarbamate fungicide; OP = organophosphorus insecticide; MIN = Mineral; EBI = Ergosterol biosynthesis inhibitor fungicide; SP = Synthetic pyrethroid CARB = carbamate insecticide

References:
a = Canadian Wildlife Service 1996. LD 50 pesticide database.Unpublished data.
b = Baril et al., 1994; c = Grolleau and Caritez 1986; d = Hudson et al., 1984;
e = Schafer et al., 1983; f = Smith 1987; g = Bradbury and Coats 1982;
h = Rattner and Franson 1984; i = Henderson et al., 1994

Acute Toxicity Index = amount (mg) of chemical to attain LD50 in a 20 g bird
Chronic Toxicity Index = amount (mg) to reach ADI in a 20 g bird
- = no LD50 data

Table 2. Residues (ug/g wet weight) measured on grasshoppers and calculated residue per unit dose for 1 kg/ha application rate of chemical

Pesticide	Grasshopper Mass (mg)	Mean Residue ug/g	Reference	Residue per unit dose (ug/g) for 1 kg/ha
Carbofuran	90	2.1	Forsyth and Westcott, 1994	15.9
Carbofuran	220	3.9	Forsyth and Westcott, 1994	29.5
Carbofuran	370	2.5	Hawley and Somers, 1998	17.9
Carbofuran	370	5.7	Hawley and Somers, 1998	40.7
Carbofuran	-	5.71 *	Leighton and Wobeser, 1987	43.2
Acephate	50	12.4	Stromberg et al., 1984	20.2
				mean = 27.9 +/- 11.8 ug/g

- information not provided in reference

* mean of two samples collected at the same site with residues of carbofuran of 4.22 and 7.2 ug/g

Table 3. Organochlorine concentrations (wet weight ug/g) in eggs of tree swallows and eastern bluebirds from study sites with nests that were not sprayed with current-use pesticides in at least one year in southern Ontario (1988-1994)

Site	Tree Swallow pp'DDE concentration	Tree Swallow Total Organochlorine concentration	% lipid	N
Orchard 1	2.56	3.47	9.04	1
Orchard 2	0.38	0.74	7.3	1
Orchard 3	0.45	0.86	7.9	1
Orchard 4	0.78	1.34	5.1	1
Orchard 5	2.06	2.51	5.45	1
Orchard 5	0.66	1.36	10.7	1
Orchard 7	0.43	1.21	6.1	1
Orchard 8	0.54	0.91	5.46	1
Old Orchard 1	1.86	2.38	6.48	1
Pasture 1	0.59	0.98	8.9	1

N= number of egg pools

* Total organochlorine concentration = sum concentration of the following compounds: 1245- and 1234- tetrachlorobenzene, pentachlorobenzene, alpha-, beta- and gamma-hexachlorocyclohexane,heptachlor epoxide, oxychlordane, trans- and cis-chlordane, dieldrin, photomirex, mirex, pp'DDE, pp'DDD, pp'DDT, op'DDE, op'DDD, and op'DDT, endosulfan, cis- nonachlor, Total PCBs

** Concentrations and % lipid are mean (standard error). All other cases are pooled analytical results.

Table 3 (CONTINUED). Organochlorine concentrations (wet weight ug/g) in eggs of tree swallows and eastern bluebirds from study sites with nests that were not sprayed with current-use pesticides in at least one year in southern Ontario (1988-1994)

Site	Eastern Bluebird pp'DDE concentration	Eastern Bluebird Total Organochlorine concentration	% lipid	N
Orchard 1	105.1(88.3)	106.3	7.7 (0.6)	4 **
Orchard 2	0.37 (0.18)	0.50	7.7 (0.05)	2 **
Orchard 3	0.64	0.88	8.00	1
Orchard 5	0.58	1.00	11.30	1
Orchard 7	0.75	0.88	5.18	1
Orchard 8	0.60	1.40	6.40	1
Orchard 9	4.87	5.26	15.94	1
Orchard 10	0.67	0.98	4.40	1
Orchard 11	1.37	1.50	3.82	1
Old Orchard 1	5.95	6.50	7.30	1
Old Orchard 2	26 (5.1)	17.10	6.6 (0.9)	2 **
Pasture 1	0.40	0.47	6.50	1

N= number of egg pools

* Total organochlorine concentration = sum concentration of the following compounds: 1245- and 1234- tetrachlorobenzene, pentachlorobenzene, alpha-, beta- and gamma- hexachlorocyclohexane,heptachlor epoxide, oxychlordane, trans- and cis-chlordane, dieldrin, photomirex, mirex, pp'DDE, pp'DDD, pp'DDT, op'DDE, op'DDD, and op'DDT, endosulfan, cis- nonachlor, Total PCBs

** Concentrations and % lipid are mean (standard error). All other cases are pooled analytical results.

Table 4. Results of testing for effect of organochlorine residues in eggs on reproduction for nests not exposed to pesticide applications during 1988-1994 and initiated before June 1stin each year

Tree Swallow	1993		1988-1994	
	Slope	P	Slope	P
Clutch Size *	0.083	0.568	0.467	0.535
Infertile + Embryo Death	-0.003	0.518	0.119	0.146
Non-hatch	0.047	0.119	0.136	0.222
Chick Death	0.002	0.460	0.082	0.331
Non-fledged Eggs	0.023	0.347	0.203	0.211
Eastern Bluebird	1992		1988-1994	
	Slope	P	Slope	P
Clutch Size	-0.189	0.277	0.080	0.804
Infertile + Embryo Death	0.168	0.111	0.112	0.014
Non-hatch	0.168	0.099	0.110	***0.008***
Chick Death	-0.076	0.634	0.112	0.098
Non-fledged Eggs	0.013	0.363	0.257	0.013

Table entries show estimated slope for a regression against log (total organochlorine concentration) and P value for a one-sided test for trend using a randomization test.

Bold - Italics P values indicate significance at sequential Bonferroni p<=0.01

* For clutch size, test is for a declining trend for all other variables test is for an increasing trend.

Non-fledged Eggs= sum of eggs that failed to hatch, chick mortality, and eggs or chicks that went missing without explanation.

Table 5. Sample sizes and cumulative toxicity scores found for tree swallows and eastern bluebirds

	Eastern Bluebird				Eastern Bluebird			
	Nest Initiation Prior to 1st June				Nest Initiation After to 1st June			
			Cumulative Toxicity Scores: (+)				Cumulative Toxicity Scores: (+)	
Year	N eggs	N nests	Mean (Std.Dev)	Maximum	N eggs	N nests	Mean (Std.Dev)	Maximum
1988	24	5	0.56 (1.21)	2.73	31	7	0.05 (0.07)	0.19
1989	98	20	0.61 (1.28)	4.14	78	20	0.83 (3.5)	15.69
1990	211	44	0.42 (0.94)	3.59	166	40	0.04 (0.07) *	0.22
1991	101	22	0.74 (1.15) **/ ++	3.77	100	26	0.11 (0.08) ++	0.28
1992	205	42	0.71 (1.22)	3.27	178	42	0.13 (0.24)	0.83
1993	158	33	0.60 (1.04)	2.85	116	27	0.09 (0.09)	0.25
1994	133	27	0.49 (1.02)	3.43	127	30	2.05 (10.6)	58.57
1988-1994	930	193	0.58 (1.09)	3.77	796	192	0.47 (4.34)	58.57

(+) Cumulative toxicity scores based on first day of pre-incubation to last day of fledging

* Significant difference between e. bluebird (nests after 1st June) and tree swallow in 1990

** Significant difference between e. bluebird (nests prior to 1st June) and tree swallow in 1991

++ Significant difference between e. bluebird (nests prior to 1st June) and e. bluebird (nests after 1st June) in 1991

*** Significant difference among years for tree swallow nests. Cumulative toxicity score was significantly higher in 1992 than in 1989, 1990, 1991.

Table 5 (CONTINUED). Sample sizes and cumulative toxicity scores found for tree swallows and eastern bluebirds

Tree Swallow
Nest Initiation Prior to 1st June

Year	N eggs	N nests	Cumulative Toxicity Scores: (+) Mean (Std.Dev)	Maximum
1988	122	24	0.65 (0.94)	2.72
1989	226	40	0.12 (0.26)	0.75
1990	183	37	0.12 (0.16) *	0.77
1991	362	69	0.13 (0.21) **	1.06
1992	723	123	0.87 (1.32) ***	3.27
1993	877	144	0.59 (0.97)	2.96
1994	105	18	0.37 (0.89)	2.80
1988-1994	2598	455	0.51 (0.97)	3.27

(+) Cumulative toxicity scores based on first day of pre-incubation to last day of fledging
* Significant difference between e. bluebird (nests after 1st June) and tree swallow in 1990
** Significant difference between e. bluebird (nests prior to 1st June) and tree swallow in 1991
++ Significant difference between e. bluebird (nests prior to 1st June) and e. bluebird (nests after 1st June) in 1991
*** Significant difference among years for tree swallow nests. Cumulative toxicity score was significantly higher in 1992 than in 1989, 1990, 1991.

Table 6. Results of linear regression and randomization analysis of reproductive results and cumulative toxicity scores for tree swallows nesting in orchards and non-sprayed sites in Ontario (1988-1994).

Parameter	Year	Background *	Intercept **	Slope	Decline ***	Estimated Significance ****	% Decline (a)
Fertility	1988	0.998	0.906	-0.519	-0.404	***0.001***	13 %
	1989	0.971	7.89	-0.551	-0.00006	0.980	
	1990	0.928	6.78	-0.215	-0.00073	***0.001***	12 %
	1991	0.961	7.73	-0.342	-0.0004	0.022	
	1992	0.910	9.96	-0.789	-0.00009	0.650	
	1993	0.950	6.14	-0.236	-0.0025	***0.002***	0.1 %
	1994	0.926	10.0	-1.28	-0.0002	***0.001***	
	1988-1994	0.936	6.38	-2.02	-0.0118	0.049	
Daily egg survival	1988	0.997	9.74	-1.56	-0.0003	0.516	
	1989	0.996	1.40	-2.62	-0.085	***0.001***	0.01 %
	1990	0.999	9.70	-0.009	-0.00006	0.940	
	1991	0.999	3.31	-0.942	-0.331	***0.001***	0.8 %
	1992	0.998	8.78	-2.16	-0.0014	***0.002***	0 %
	1993	0.998	7.57	-0.052	-0.0005	***0.002***	0.18 %
	1994	0.999	9.87	-0.261	-0.00007	1.00	
	1988-1994	0.999	5.05	-0.374	-0.0009	0.037	

Table 6 (CONTINUED). Results of linear regression and randomization analysis of reproductive results and cumulative toxicity scores for tree swallows nesting in orchards and non-sprayed sites in Ontario (1988-1994).

Parameter	Year	Background *	Intercept **	Slope	Decline ***	Estimated Significance ****	% Decline (a)
Daily chick survival	1988	0.989	10.0	-4.98	-0.007	0.018	
	1989	0.998	3.44	-0.134	-0.0298	***0.001***	14 %
	1990	0.996	8.80	-5.00	-0.00004	1.00	
	1991	0.996	5.79	-1.18	-0.002	0.028	
	1992	0.978	8.41	0.000	-0.0002	0.204	
	1993	0.999	6.05	-1.45	-0.011	***0.001***	0.3 %
	1994	0.998	4.33	-0.089	-0.0140	***0.001***	8 %
	1988-1994	0.997	4.95	-0.008	-0.007	0.205	
Clutch Size	1988	NA	6.51	0.020	NA	0.982	
	1989	NA	5.90	0.029	NA	0.622	
	1990	NA	6.00	0.142	NA	0.981	
	1991	NA	5.11	-0.015	NA	0.330	
	1992	NA	5.88	0.001	NA	0.50	
	1993	NA	6.17	0.013	NA	0.758	
	1994	NA	6.06	0.027	NA	0.532	
	1988-1994	NA	6.19	0.058	NA	0.958	

Notations to Table 6:

* Background = simulated survival rate under no exposure
** Intercept = point at which the curve approaches 50% decline
****Estimated significance based on 1000 randomisations;
Bold-Italic values indicate p values which are significant based on sequential Bonferroni analysis with p<=0.016
***Difference between probability at background and at highest observed exposure
NA = Not applicable
(a) % Decline value is the % reduction in egg fertility or egg or chick survival based on known mean cumulative toxicity score divided by the no. days of exposure for each reproductive category ie. 12 days for egg fertility; 13 days for egg incubation; 20 days for chick survival

Table 7. Results of linear regression and randomization analysis of reproductive results and cumulative toxicity scores for eastern bluebirds in orchards and non-sprayed sites in Ontario (1988-1994).

Eastern Bluebird nests initiated prior to 1st June in each year

Parameter	Year	Background *	Intercept **	Slope	Decline ***	Estimated Significance ****	% Decline (a)
Fertility	1988	--	--	--	--	--	
	1989	0.999	6.51	-4.12	-0.083	***0.013***	0 %
	1990	0.960	2.62	-0.035	-0.067	0.076	
	1991	0.964	9.02	-1.01	-0.0004	0.569	
	1992	0.883	6.64	-5.83	-0.279	0.021	
	1993	0.980	4.36	-0.004	0.012	0.062	
	1994	0.951	6.50	-0.939	-0.004	0.111	
	1988-1994	0.955	3.25	-0.077	-0.038	0.026	
Daily egg survival	1988	--	--	--	--	--	
	1989	0.998	9.01	-0.931	-0.00045	0.402	
	1990	0.998	9.84	-0.527	-0.0001	0.869	
	1991	0.999	7.13	0.078	0.0007	0.57	
	1992	0.997	7.84	-2.52	-0.0062	0.222	
	1993	0.996	7.38	-3.27	-0.0169	0.132	
	1994	0.998	9.14	-5.00	-0.0166	0.062	
	1988-1994	0.997	9.97	-3.97	-0.0123	0.123	

Table 7 (CONTINUED). Results of linear regression and randomization analysis of reproductive results and cumulative toxicity scores for eastern bluebirds in orchards and non-sprayed sites in Ontario (1988-1994).

Eastern Bluebird nests initiated prior to 1st June in each year

Parameter	Year	Background *	Intercept **	Slope	Decline ***	Estimated Significance ****
Daily chick survival	1988	--	--	--	--	--
	1989	0.997	4.51	-0.154	-0.0133	0.271
	1990	0.981	8.74	-4.99	-0.0856	0.076
	1991	0.999	8.96	-0.095	-0.00014	0.517
	1992	0.987	9.99	-0.573	-0.00009	0.863
	1993	0.971	10.0	-0.541	-0.00008	0.91
	1994	0.978	8.08	-2.82	-0.0097	0.332
	1988-1994	0.983	9.97	-3.48	-0.0064	0.260
Clutch Size	1988	--	--	--	--	--
	1989	NA	5.55	0.08	NA	0.992
	1990	NA	4.73	-0.007	NA	0.399
	1991	NA	5.60	0.069	NA	0.853
	1992	NA	4.90	0.003	NA	0.508
	1993	NA	5.04	0.032	NA	0.735
	1994	NA	4.73	-0.021	NA	0.319
	1988-1994	NA	4.94	0.016	NA	0.810

Notation for Table 7:

* Background = simulated survival rate under no exposure
** Intercept = point at which the curve approaches 50% decline
****Estimated significance based on 1000 randomizations
***Difference between probability at background and at highest observed exposure
Bold-Italic values indicate p values which are significant based on sequential Bonferroni analysis with p<=0.016
-- N=5 nests; sample size too small for statistical analysis
(a) % Decline value is the % reduction in egg fertility or egg or chick survival based on known mean cumulative toxicity score divided by the no. days of exposure for each reproductive category ie. 12 days for egg fertility; 13 days for egg incubation; 20 days for chick survival

Table 8. Results of linear regression and randomization analysis of reproductive results and cumulative toxicity scores for eastern bluebirds in orchards and non-sprayed sites in Ontario (1988-1994).

Eastern Bluebird nests initiated after 1st June in each year

Parameter	Year	Background *	Intercept **	Slope	Decline ***	Estimated Significance ****	% Decline (a)
Fertility	1988	--	--	--	--	--	
	1989	0.8221	8.420	-4.049	-0.0000	0.910	
	1990	0.8860	7.350	-5.93	-0.0000	1.00	
	1991	0.8037	7.350	-2.114	-0.00002	0.703	
	1992	0.9695	10.0	0.757	0.0784	0.887	
	1993	0.999	9.944	0.738	0.00032	0.979	
	1994	0.9979	2.0148	-0.0526	-0.1357	***0.001***	4.0%
	1988-1994	0.9176	9.9643	0.6907	0.0411	0.868	
Daily egg survival	1988	--	--	--	--	--	
	1989	0.994	1.560	-5.00	-0.00002	0.433	
	1990	0.999	6.533	-1.793	-0.00005	0.997	
	1991	0.999	-1.253	-4.978	-0.00238	0.044	
	1992	0.999	6.541	0.198	0.00888	0.963	
	1993	0.999	-5.65	-4.999	-0.10755	***0.001***	0 %
	1994	0.999	9.82	-0.3461	-0.00022	***0.001***	0.04%
	1988-1994	0.998	7.065	0.980	0.00169	0.885	

Table 8 (CONTINUED). Results of linear regression and randomization analysis of reproductive results and cumulative toxicity scores for eastern bluebirds in orchards and non-sprayed sites in Ontario (1988-1994).

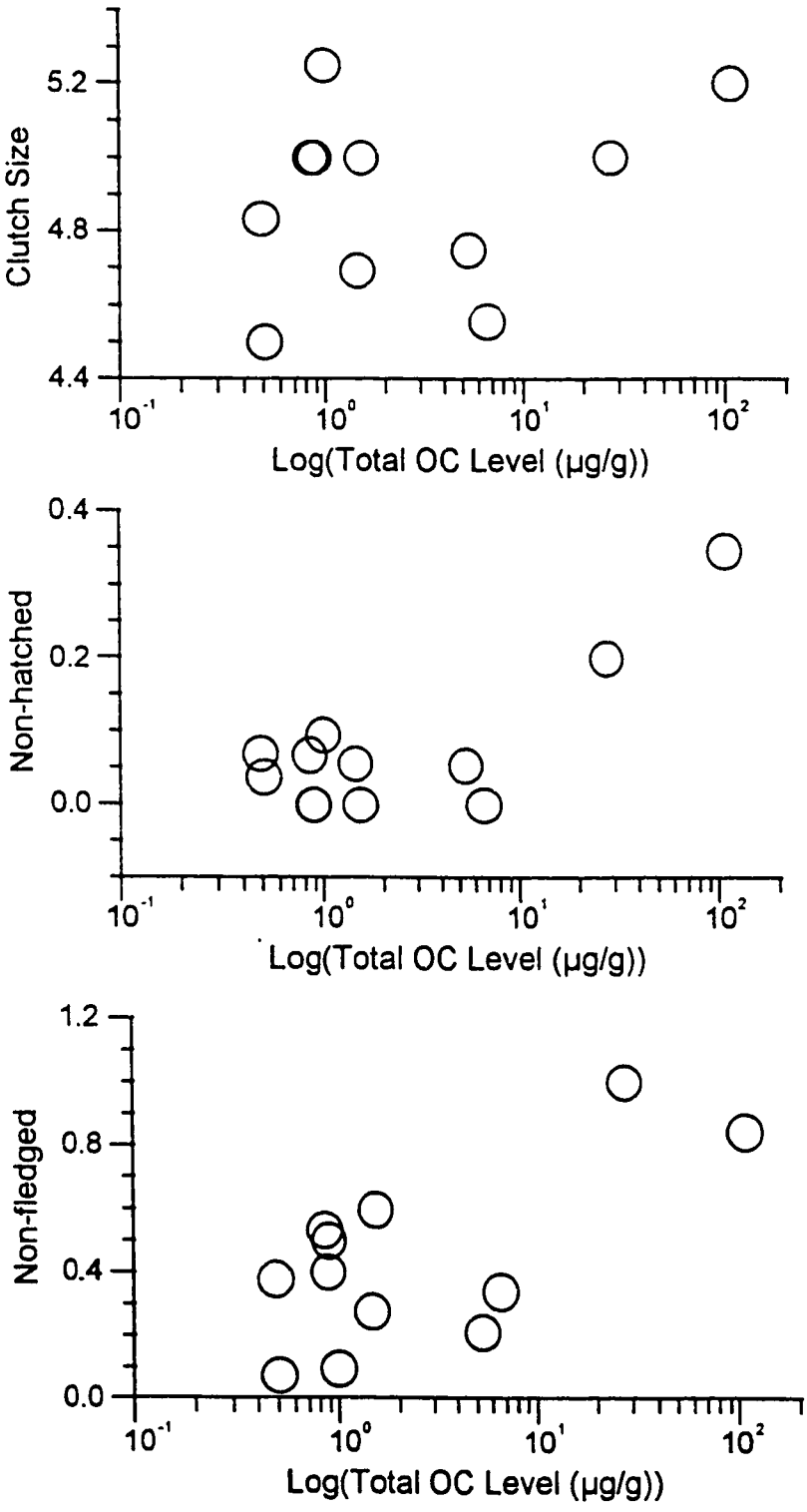
Eastern Bluebird nests initiated prior to 1st June in each year

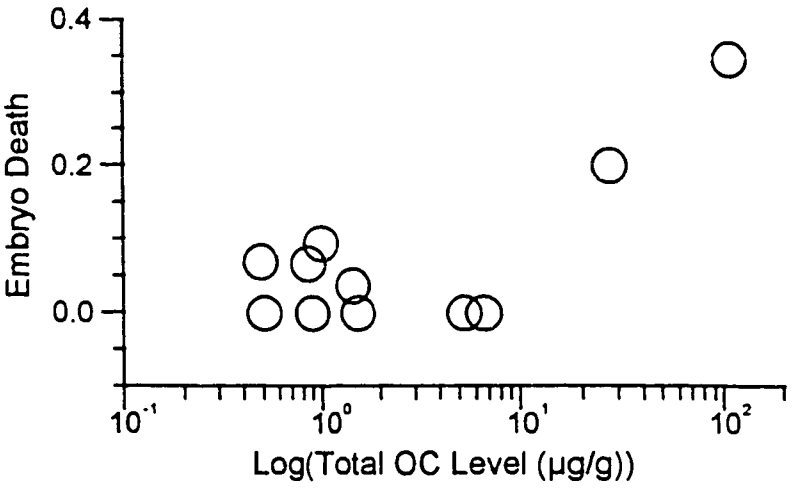
Parameter	Year	Background *	Intercept **	Slope	Decline ***	Estimated Significance ****	% Decline (a)
Daily chick survival	1988	--	--	--	--	--	
	1989	0.991	9.866	-0.1105	-0.00007	0.756	
	1990	0.993	8.834	-3.991	-0.0000	1.00	
	1991	0.994	9.897	-1.489	-0.00001	0.821	
	1992	0.999	4.709	-0.145	-0.00661	***0.010***	1.9%
	1993	0.989	3.879	-4.403	-0.00006	0.262	
	1994	0.998	5.953	-0.069	-0.00342	***0.001***	5.7%
	1988-1994	0.995	5.812	-0.0588	-0.00212	0.255	
Clutch Size	1988	--	--	--	--	--	
	1989	NA	3.51	-0.049	NA	0.253	
	1990	NA	4.10	-0.005	NA	0.493	
	1991	NA	4.10	0.039	NA	0.628	
	1992	NA	3.66	-0.072	NA	0.051	
	1993	NA	4.85	0.074	NA	0.875	
	1994	NA	4.73	-0.021	NA	0.319	
	1988-1994	NA	3.91	-0.03	NA	0.063	

Notation for Table 8:

* Background = simulated survival rate under no exposure
** Intercept = point at which the curve approaches 50% decline
****Estimated significance based on 1000 randomizations
***Difference between probability at background and at highest observed exposure
Bold-Italic values indicate p values which are significant based on sequential Bonferroni analysis with p<=0.016
-- N=5 nests; sample size too small for statistical analysis
(a) % Decline value is the % reduction in egg fertility or egg or chick survival based on known mean cumulative toxicity score divided by the no. days of exposure for each reproductive category ie. 12 days for egg fertility; 13 days for egg incubation; 20 days for chick survival

Figure 1. Total organochlorine concentration in eastern bluebird eggs and reproductive parameters in nests not sprayed with chemicals in 1988-1994 and initiated prior to 1st June.


Clutch Size
5.2
4.8
4.4
10^{-1}
10^{0}
10^{1}
10^{2}
Log(Total OC Level (µg/g))
Non-hatched
0.4
0.2
0.0
10^{-1}
10^{0}
10^{1}
10^{2}
Log(Total OC Level (µg/g))
Non-fledged
1.2
0.8
0.4
0.0
10^{-1}
10^{0}
10^{1}
10^{2}
Log(Total OC Level (µg/g))


0.4
0.2
0.0
Embryo Death
10^{-1}
10^{0}
10^{1}
10^{2}
Log(Total OC Level (µg/g))

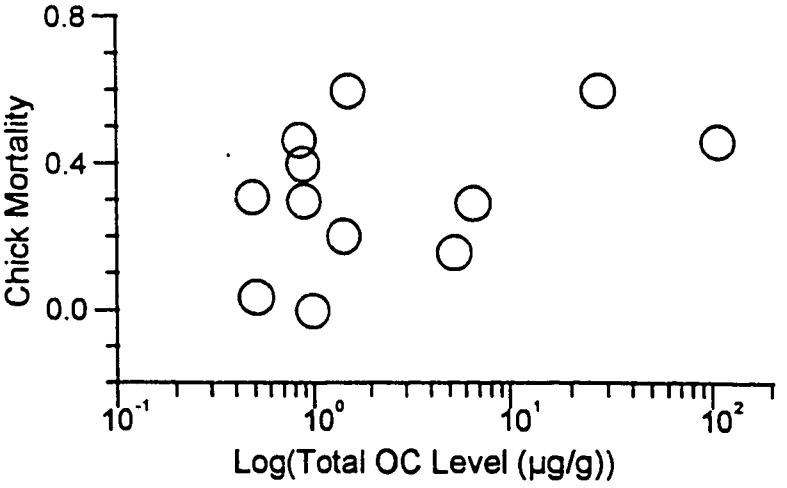

0.8
0.4
0.0
Chick Mortality
10^{-1}
10^{0}
10^{1}
10^{2}
Log(Total OC Level (µg/g))

Figure 2. Cumulative toxicity scores and total organochlorine concentrations in tree swallow eggs for nests initiated prior to 1st June. The number of petals on the sunflower indicates the number of observations in the grid block.

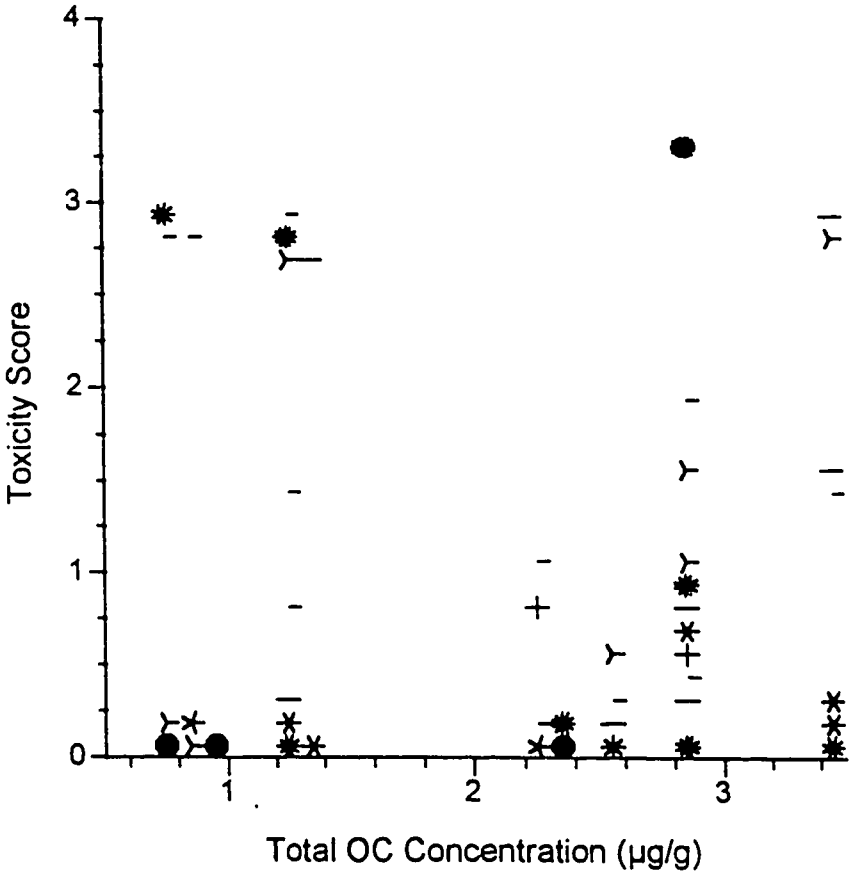

Toxicity Score
0
1
2
3
4
1
2
3
Total OC Concentration (µg/g)

Figure 3. Cumulative toxicity scores and total organochlorine concentrations in eastern bluebird eggs for nests initiated prior to 1st June. The number of petals on the sunflower indicates the number of observations in the grid block.

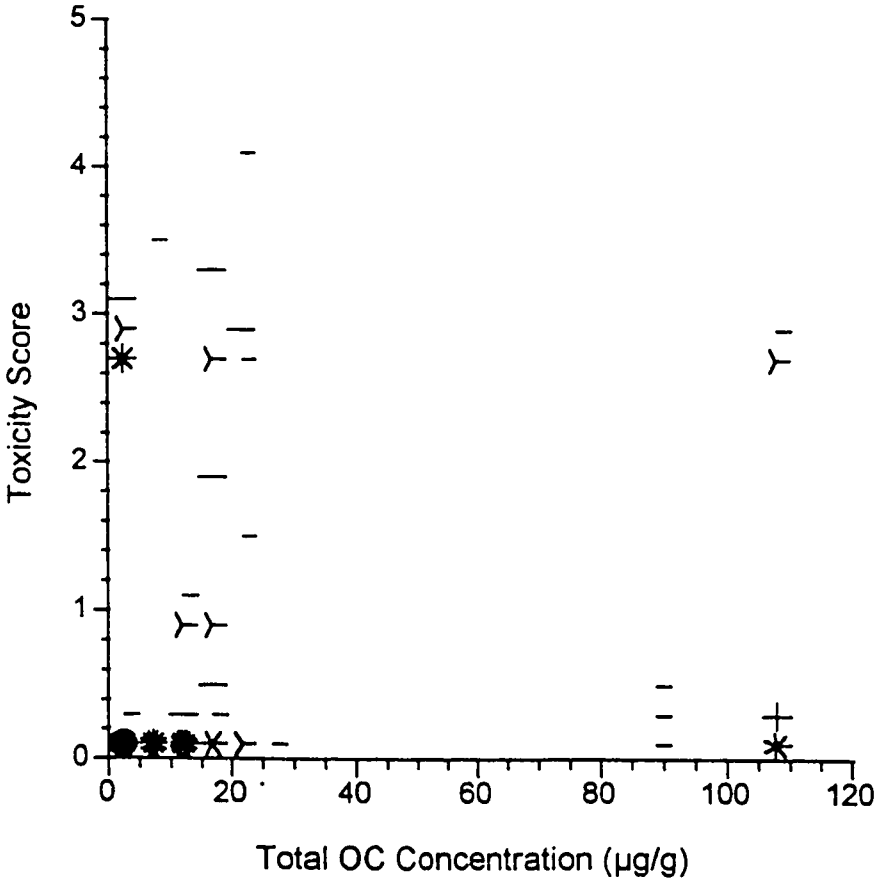
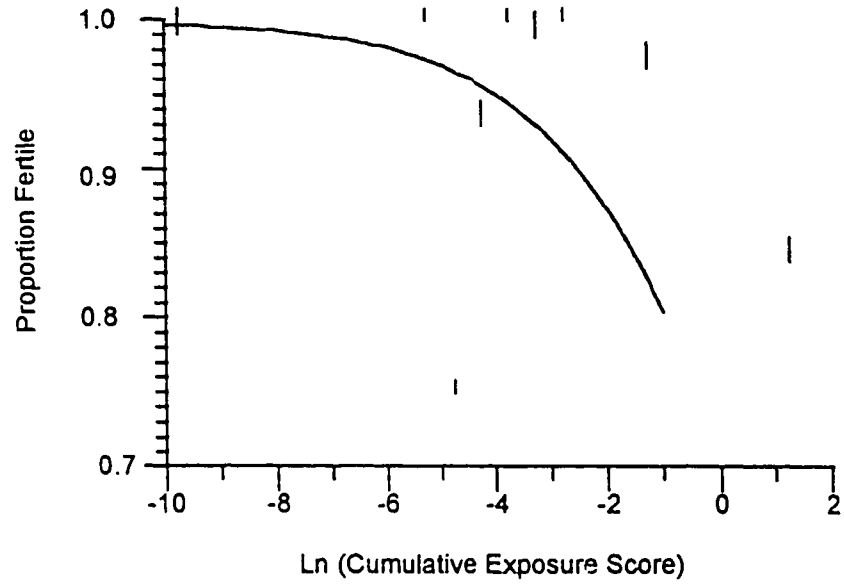

Toxicity Score
0
1
2
3
4
5
0
20
40
60
80
100
120
Total OC Concentration (µg/g)

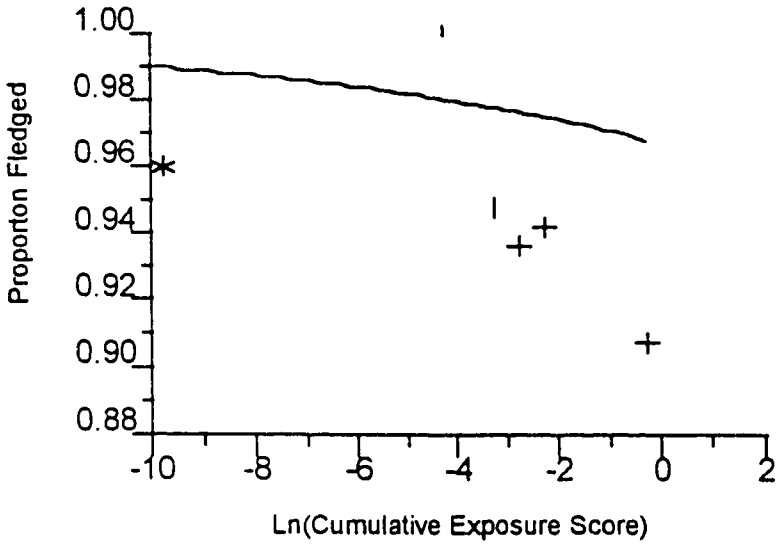
Figure 4. Tree swallow reproduction and cumulative toxicity scores for (a) 1988 (b) 1989

(a) 1988



The following symbols represent these numerical values on each graph:	ı - 1-10 | - 11-50 ⅄ - 51-100	+ - 101-500 * - 501-1000 * - > 1000
Symbols represent number of eggs.		

(b) 1989



The following symbols represent these numerical values on each graph:	ı - 1-10 | - 11-50 ⊥ - 51-100	+ - 101-500 ★ - 501-1000 ✱ - > 1000

Symbols represent days at risk.

CONCLUSIONS

This study found significant effects of pesticide applications in apple orchards on reproduction and health in tree swallows and eastern bluebirds. Both tree swallows and eastern bluebirds experienced significant declines in reproduction with increasing exposure and toxicity of pesticides applied. In particular, egg fertility and survival were affected. Although the pesticide exposure was similar, tree swallows were more sensitive than eastern bluebirds to the effects of pesticides applied during 1988-1994. Also, the effects on eastern bluebirds of pesticides used during 1988-1994 may have been exacerbated by the presence of organochlorines in bluebird eggs which were also associated with unhatched eggs. Notably, this is the first time that increasing concentrations of organochlorine pesticides in eggs of wild passerines have been reported to correlate with decreased egg hatching success.

Of the tree swallow chicks that survive to fledge, the immune and endocrine systems exhibit significant changes when exposed to typical pesticide exposures in apple orchards. In sprayed birds, there was a significantly increased blastogenic response to pokeweed mitogen, increased thymocyte proliferation in the cortex and delayed thymic involution. Sprayed birds were also slightly anemic compared to birds from non-sprayed sites and possessed smaller bursal masses and an increase in relative heterophil concentrations. Local bursal inflammation was also found in sprayed birds but may have been caused by trematode parasite infections although pesticide exposure also correlated positively with this condition. This was the first study of the immunology and effects of current pesticide exposures in wild passerines therefore it is difficult to predict the long-term consequences of the stimulated immune systems in sprayed birds. However, some environmental contaminants that overtly stimulate the immune system in mammals have induced hypersensitivity and/or autoimmunity and such an effect may occur in tree swallows.

In the endocrine system of 16-day old male tree swallow chicks, the concentration of the thyroid hormone tri-iodo-L-thyronine (T3) positively correlated with the number of mixtures of sprays applied during egg incubation through chick-rearing. There was also a trend between increasing occurrence of a disrupted sertoli cell population on the seminiferous tubular basement membranes as the number of mixtures of pesticides sprayed during chick-rearing increased. Correlations with spray exposure were found in only male chicks suggesting that developing male tree swallow embryos are the most sensitive to the effects of pesticides. This is consistent with other studies that indicated the high sensitivity of developing male mammals to environmental contamination (Colborn and Clement 1992).

The study of behaviour of tree swallows exposed to pesticides showed that there were short-term behavioural effects on the ability of adult birds to deliver food but no effect on incubating activity. The begging of chicks was exacerbated in the orchards but this appeared to be associated with chronically low food availability in the orchards rather than changes in behaviour induced by pesticide exposure. Although behavioural changes were found, ultimately, the weights of the chicks produced in the orchards were unaffected. This suggests that effects on reproduction, immune and endocrine systems are probably the result of direct impacts of pesticides on eggs and chicks rather than an indirect effect of abnormal behaviour or removal of food resources causing poor body condition in fledging birds.

The implication of this field study is that birds developing in nests in agricultural areas are at significant risk to their health and the survival due to the pesticide exposure. While the organophosphorus and carbamate pesticides present the most acutely toxic threat to the birds, the fungicides that are sprayed alone or in combination with these chemicals are actually sprayed more often than the insecticides and studies suggest that these fungicides can have sublethal health effects on vertebrates consistent with the effects found in tree swallows. It is now important to define

whether the effects found here are due to a specific individual compound, a mixture of these compounds or the overall chronic exposure to many compounds throughout the nesting seasons. It should also be determined if these effects are induced when exposures occur at specific periods of development in the birds. This information is critical to developing management options for pesticide use in apple orchards which will be effective for pest control yet protect the health of songbirds. Unfortunately, there were no orchards in Ontario in which fungicides or insecticides were exclusively applied therefore the individual effect of these compounds on reproduction, immune and endocrine function could not be derived from this type of study. Nonetheless, the field approach and its findings validate the need to identify the chemicals or mixtures of chemicals causing the problems. If this research had not found any differences between sprayed and non-sprayed birds, there would be no justificiation for the expense and time required for further testing of the effects of individual pesticides and/or the combined effects of pesticides used in apple orchards.

References

Colborn, T., and Clement, C. 1992. Chemically-induced alterations in sexual and functional development: the wildlife/human connection. Advances in Modern Environmental Toxicology ed. Mehlman, M.A. Vol. XXI. Princeton Scientific Publ. Co. Inc. Princeton, NJ. 403 pp.