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Effects of Azinphos-Methyl on Cholinergic Responses and General Health in Zebra Finches (*Taeniopygia guttata*) After Previous Treatment with *p,p′*-DDE

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Abstract. Although organochlorine (OC) pesticides were replaced with organophosphates (OPs) in the early 1970s, they continue to persist in orchard environments today. Extensive research has been conducted to determine the effects of currently used OPs on cholinesterase (ChE) activity; however, although OCs continue to be prevalent in areas of previous use, few studies have looked at the toxicity of a combination of residual OC compounds with currently used OP pesticides. The focus of our study was to determine the effects of azinphos-methyl (a common OP used in apple orchards today) on ChE activity and general health in zebra finches (*Taeniopygia guttata*) previously exposed to *p,p′*-DDE (a commonly detected metabolite of DDT). The main results of our study were as follows: (1) azinphos-methyl alone caused a dose-dependent inhibition of plasma and brain ChE activity; (2) *p,p′*-DDE in combination with azinphos-methyl did not change azinphos-methyl inhibition of ChE activity; and (3) there were suggestions of immunostimulation in birds dosed 1 year previously to *p,p′*-DDE and of anemia when *p,p′*-DDE was combined with azinphos-methyl; however, there was no dose–response for these parameters in birds subsequently dosed with *p,p′*-DDE.

Azinphos-methyl (O,O-dimethyl S-[(4-oxo-1,2,3-benzo-triazin-3(4H)-yl)methyl] phosphorodithioate) is a broad-spectrum organophosphate (OP) insecticide used extensively throughout the world to control insect pests (Chemagro Division Research Staff 1974). In Canada, azinphos-methyl is registered for control of insects on agricultural and vegetable crops, primarily apples, peaches, potatoes, and cherries. In apple orchards, azinphos-methyl is commonly used to control infestation of the insect pest, coddling moth (*Cydia pomonella*), and is sprayed one to three times per season (Province of British Columbia 1991).

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Like all OP insecticides, azinphos-methyl acts by inhibiting the cholinesterase (ChE) enzymes (Chambers and Levi 1992). Those enzymes are involved in the breakdown of the neurotransmitter acetylcholine to acetate and choline, thus providing a key role in the removal of excess acetylcholine. ChE enzymes are critical to the normal function of the nervous system in both vertebrates and invertebrates, and therefore the potential exists for nontarget effects whenever OPs are released into the environment (Grue *et al.* 1991). Measurement of these enzymes is commonly used in field situations to determine the cause of mortality or extent of exposure to OPs (Zinkl *et al.* 1980; Busby *et al.* 1981). Several field-monitoring studies have measured ChE inhibition to examine the response of songbirds residing in orchards after azinphos-methyl treatment (Graham and DesGranges 1993; Burgess *et al.* 1999; Gill *et al.* 2000).

Although no longer applied, organochlorine (OC) pesticides, particularly DDT [1,1,1-trichloro-2,2-*bis*(*p*-chlorophenyl)ethane]–related compounds, persist at high concentrations in areas of past intensive use, such as fruit orchards (Harris *et al.* 2000). Songbirds residing in Ontario and British Columbia fruit orchards have been reported to contain high body burdens of DDE [2,2- *bis*(4-chlorophenyl)-1,1-dichloroethylene, a persistent metabolite of DDT]. Eastern bluebird (*Sialia sialis*) eggs from Ontario orchards had *p,p′*-DDE residues as high as 105 μg/g (Bishop *et al.* 2000) and DDE concentrations in American robin (*Turdus migratorius*) eggs in Okanagan orchards (British Columbia) were as high as 302 μg/g (Gill *et al.* 2003). Persistence of DDT and its metabolites in orchard soils has been suggested to be caused by high previous application rates and minimal tillage or other soil disturbance, which limit loss by volatilization and erosion (Harris *et al.* 2000).

Because of the persistence of DDE in the environment, songbirds residing in orchards commonly have high DDE (particularly *p,p′*-DDE) body burdens when OP spraying occurs. However, few avian studies have examined the joint toxicity of OC and OP pesticides. In mammals, previous dosing or exposure to OC compounds appears to decrease the effects of subsequent OP exposure by increasing activity of the ChE enzymes (Ball *et al.* 1954; Triolo and Coon 1966; Menzer

1970). However, it appears this protection is not seen in avian species. For example, Ludke (1977) found that DDE exposure in coturnix quail (*Coturnix coturnix japonica*) resulted in a dose-related increase in plasma ChE activity. After treatment with the OP parathion, plasma ChE activity decreased in control and DDE-pretreated birds to approximately the same levels. Because DDE-pretreated birds had relatively greater plasma ChE activity before parathion dosage, dosing with parathion resulted in a relatively greater ChE inhibition in DDE pretreated birds than untreated individuals. Thus, in quail, previous feeding of DDE did not decrease the effect of subsequent parathion exposure (see also Dieter 1974).

In addition to enzyme effects, the general health of animals exposed to environmental stressors such as pesticides may be negatively impacted. One of the first field studies to examine immune parameters in wild birds exposed to pesticides found a general trend of immunostimulation in tree swallows (Bishop *et al.* 1998). Basal hematologic parameters, such as percent hematocrit and leucocrit, are easily obtainable and are useful for detecting the effects of environmental, infectious, parasitic, or toxicologic stresses on animals. They have been widely used in studies of health (Ots *et al.* 1998), reproduction (Morton 1994; Merila and Svensson 1995), and adaptation (Carpenter 1975).

The objectives of this study were (1) to determine the dose–response relationship between azinphos-methyl and brain and plasma ChE activity in the zebra finch (*Taeniopygia guttata*) and (2) to determine the effects of azinphos-methyl on ChE activity and hematologic and immune-function parameters in birds pre-exposed to *p,p′*-DDE at low, medium, and high doses. Our study was part of a larger laboratory investigation of the sublethal effects of orchard pesticides on songbird reproduction.

Methods

Animals and Husbandry

This study was conducted on a captive colony of zebra finches maintained at the Simon Fraser University Animal Care Facility located in Burnaby, British Columbia. Zebra finches were housed in a controlled environment (temperature 19°C to 23°C; humidity 35% to 55%; photoperiod 14 hours light to 10 hours dark; lights on at 0700). All birds were provided with mixed seed (panicum and white millet 1:2; 11.7% protein, 0.6% lipid, and 84.3% carbohydrate by dry mass), water, grit, and cuttlefish bone (calcium) *ad libitum* plus a multivitamin supplement in the drinking water once per week. Experiments and animal husbandry were carried out under a Simon Fraser University Animal Care Committee permit in accordance with guidelines from the Canadian Committee on Animal Care.

Experiment 1. Dose-response of plasma and brain ChE activity to azinphos-methyl. To determine the response of brain ChE activity to azinphos-methyl, a dose–response study was conducted. A series of dilutions for azinphos-methyl dissolved in corn oil were prepared on a log scale, and the following dosages were administered after 1 hour of fasting: 0.27, 0.48, 0.85, 1.5, 2.7, and 4.8 mg/kg. One group was also exposed to the same amount of plain corn oil containing no azinphos-methyl. Three hours after dosing, birds were anesthetized and exsanguinated. Because of the low response over this range (see Results section), we subsequently dosed additional groups of birds at 14.6, 25.5, and 45.3 mg/kg per the same methodology. Three zebra finches were dosed per treatment, except for the highest dose (45.3 mg/kg) in which 5 birds were dosed. To determine the effect that duration of fasting had on brain ChE activity, we dosed 9 birds (3 birds/treatment) at the following dosages after overnight fast: 1.5, 4.8, and 8.5 mg/kg. To determine any behavioral effects, an additional 12 birds (3 birds/treatment) were fasted overnight and dosed at 1.5, 4.8, 8.5, and 14.6 mg/kg. Those birds were observed for 1 week after dosing and checked daily for mortality or abnormal behavior (such as tremors, convulsions, or lethargy).

Table 1. Summary of pesticide history and treatment in male zebra finches used to determine cholinesterase activities in birds exposed to a combination of *p,p′*-DDE and the OP azinphos-methyl

Previous Exposure to *p,p′*-DDE[a]	n	This Experiment: *p,p′*-DDE Dosage (mg/kg)	This Experiment: Azinphos-methyl Exposure
No	10	No	No
No	10	No	Yes
Yes	10	No	Yes
Yes	10		Yes
Yes	10		Yes
Yes	10		Yes

[a] Previous exposure to *p,p′*-DDE occurred at 34 mg/kg approximately 1 year before the start of the experiment.
OP = organophosphate.

The effect of azinphos-methyl exposure on plasma ChE activity was also determined. For this study, an additional 55 zebra finches (5 birds/treatment) were dosed with azinphos-methyl (or only plain corn oil for controls) after 1 hour of fasting, and plasma samples were collected for ChE activity measurement. Birds received the following dosages of azinphos-methyl: 0.27, 0.48, 0.85, 1.5, 2.7, 4.8, 8.5, 14.6, 25.5, and 45.3 mg/kg. One treatment group was also exposed to plain corn oil containing no azinphos-methyl. Three hours after dosing, birds were anesthetized, and blood samples were collected (200 µl) from the jugular vein followed by exsanguination.

Experiment 2. Effects of *p,p′*-DDE and azinphos-methyl on ChE activity and general health. Sixty adult male zebra finches were divided into cages with 5 birds cage (total of 12 cages). Because of limitations in bird numbers, 40 of the birds used were from a previous experiment in which they were exposed to *p,p′*-DDE (34 mg/kg) approximately 1 year previously. There was a total of 6 treatment groups with 10 birds/treatment (Table 1). Birds exposed to a low, medium, and high dose of *p,p′*-DDE were treated with 19, 34, and 60 mg/kg *p,p′*-DDE (log-scale dilution), respectively, daily for 4 weeks, and the remaining birds were exposed to plain corn oil daily for 4 weeks (see Dosing Procedure section).

The phytohemagglutinin (PHA) immune function test was conducted after 3 weeks of *p,p′*-DDE dosing. The PHA immune function test is now being recognized as a useful tool to study cell-mediated immunity of wild animals (Smits *et al.* 1996; Smits and Williams 1999). This test measures the response to the plant lectin PHA in terms of T-lymphocyte proliferation. For zebra finches, this measurement required that a 1-cm patch of skin on the mid-patagium of both wings be plucked of feathers. Three measurements of patagium thickness of the wings were measured to 0.01 mm using a gauge micrometer (The Dyer Company, Lancaster, PA) and the results averaged. Each bird was then injected with 30 µl PHA lectin solution in the left wing and 30 µl of phosphate-buffered saline in the right wing. Twenty-four hours after injection, thickness of both wings was

measured at the injection site. The response was considered to be the difference between the change in thickness of the PHA-injected site and that of the PBS-injected site in each bird.

Twenty-four hours after their last *p,p′*-DDE (or corn oil) dose (after 4 weeks), birds were exposed to azinphos-methyl at 18.4 mg/kg. This dosage was predicted to cause 20% to 25% brain ChE inhibition (see Results section), which is indicative of OP exposure (Ludke *et al.* 1975; Busby *et al.* 1981). Three hours after dosing, birds were anesthetized, and plasma and brain samples were collected for determination of ChE activity. In addition, blood samples (40 µl) were collected to determine percent hematocrit and leucocrit. Hematocrit, or packed cell volume, measures the relative amount of red blood cells in total blood volume and reflects the extent and efficiency of oxygen uptake and transfer to tissues (Ots *et al.* 1998). Measurement of the leucocrit (or 'buffy-coat' layer) is indicative of the number of white blood cells in a sample (Wardlaw and Levine 1983), which is one measure of humoral immune function. Within 4 hours of collection, blood was centrifuged for 3 minutes at 5,000 rpm after which the height of the buffy layer, hematocrit, and total sample volume was immediately measured using a digital caliper (±0.01 mm). The same person measured all samples.

To determine the amount of residues remaining in zebra finches exposed to *p,p′*-DDE approximately 1 year previously, three livers were submitted for OC content determination. In addition, to confirm exposure of birds treated with low, medium, and high *p,p′*-DDE doses, livers were collected from two birds from each dosage group after completion of 4 weeks of dosing. All livers were collected using acetone rinsed utensils and stored in acetone–hexane triple-rinsed jars. Tissues were immediately frozen at –20°C until analysis.

Dosing Procedure

Technical-grade azinphos-methyl (Guthion, Supelco, Bellefont, PA; 100% purity) was used in all experiments. The insecticide was dissolved in corn oil and administered by way of intubation to individual birds. Each bird received 0.1 ml insecticide–corn oil solution (or plain corn oil for controls). Birds were fasted for 1 hour before dosing. To determine the effect that duration of fasting had on birds, we dosed an additional subsample of birds after overnight fast. Dosages were calculated based on the average weight per bird, which was 15.0 ± 0.3 g (mean ± standard error). Birds were given food and water 10 to 15 minutes after dosing.

Three hours after dosing with azinphos-methyl or corn oil, birds were anesthetized via intramuscular injection of 50 µl ketamine and xylazine solution (50:50 by volume; Associated Veterinary Products, Abbotsford, British Columbia) and then exsanguinated. Blood and brain samples were collected within 2 minutes of death. Blood samples were collected via the jugular vein using heparinized pipettes and transferred to heparinized 0.6-ml centrifuge tubes. Heads were separated from bodies and stored in sterile plastic bags. Both blood and heads were immediately stored on ice for up to 6 hours. Plasma was separated from the red blood cell component by centrifugation at 5,000 rpm for 15 minutes and immediately frozen at –20°C. Heads were frozen at –20°C within 6 hours of collection.

Technical grade DDE [*p,p′*-DDE; 2,2-bis(4-chlorophenyl)-1,1-dichloroethelene; Sigma-Aldrich, Oakville, Ontario; 99% purity] was used for the second study in which birds were exposed to *p,p′*-DDE before OP dosing. Birds in this study received a supplemental diet of egg food, which was the vehicle for the insecticide. Egg food consisted of a mixture of hard-boiled eggs (three eggs at approximately 60 g/egg), breadcrumbs (40 g), and corn meal (40 g), which the birds readily consumed (20.3% protein to 6.6% lipid). The pesticide was initially dissolved in corn oil, and then the insecticide–corn oil mixture (or plain corn oil for controls) was incorporated into the egg food. Three grams of prepared egg food was offered daily per bird, most of which was consumed after 24 hours. Each cage was offered with a minimum of three dishes containing the prepared egg food to minimize interbird variation in amount of food ingested. Before any pesticide exposure, birds were weighed. Dosages prepared contained 19, 34, and 60 mg/kg *p,p′*-DDE (log-scale dilution). To validate the methodology used to prepare the contaminated egg food, two food samples from each dosage (excluding 34 mg/kg) were collected randomly throughout the study and analyzed for OC content. Two food samples from a previous study prepared at 34 mg/kg *p,p′*-DDE were also analyzed for OCs. Levels for these two samples are reported in this study because the preparation methodology was the same. On collection, food samples were immediately frozen at –20°C until analysis.

Cholinesterase Assay

Head and plasma samples were assayed for ChE activity at the National Wildlife Research Centre (NWRC) in Hull, Quebec. On arrival, brains were removed from the cranium; the whole brain was homogenized in buffer/Triton-X-100 at a ratio of 250 mg/ml; and 10 µl of the homogenate was analyzed. Cholinesterase activity was determined based on Hill and Flemming's (1982) modification of the method of Ellman *et al.* (1961). The cuvette contained 3 ml DTNB [5,5′-dithiobis-(nitrobenzoic acid); 2.5 × 10-4 M] in phosphate buffer (0.05 M, pH 7.9), 10 µl brain homogenate (or 20 µl plasma); and 100 µl acetylthiocholine iodide (0.032 M for brain and 0.156 M for plasma). Cholinesterase activity was determined with a spectrophotometer (Hewlett Packard Diode Array HP8452, S/N 2610A00276) at 30°C. The change in absorbance at 406 nm during 1 minute was recorded with readings taken every 15 seconds. Duplicate analyses were performed for most samples. For each sample, the average of the duplicate assays was used in the data analyses. Quality-assurance procedures included the analysis of Precinorm E (control serum from Boehringer Mannheim) with each series of samples and conducting duplicate analyses. Three plasma samples from experiment 2 could not be analyzed because of high lipid content.

OC Analysis

Food and liver samples were analyzed at the Great Lakes Institute for Environmental Research at the University of Windsor in Windsor, Ontario for OC content. Because of insufficient sample size, percent moisture was not determined in liver samples. All solvents used during sample extractions were of high purity suitable for gas chromatography–pesticide analysis. 1,3,5-Tribromobenzene, used as a surrogate standard for analyte recovery during sample extractions, was obtained from Accu Standards (CT). OC pesticide standards containing 1,2,4,5-tetrachorobenzene, 1,2,3,4-tetrachlorobenzene, pentachlorobenzene, hexachlorobenzene, α-hexachlorocyclohexane, β-hexachlorocyclohexane, γ-hexachlorocyclohexane, octachlorostyrene, oxy-chlordane, gamma(trans)-chlordane, alpha(cis)-chlordane, trans-nonachlor, cis-nonachlor, *p,p′*-DDE, *p,p′*-DDD, *p,p′*-DDT, mirex, and photomirex were donated to GLIER by the Canadian Wildlife Service, Environment Canada, Quebec Canada. The laboratory at GLIER is accredited by the Canadian Association for Environmental Analytical Laboratories through participation in interlaboratory comparisons and routine audits.

Statistical Analyses

Statistical analyses were performed using JMP software program (SAS Institute, Inc. 2000). All parameters were tested for normality

(Shapiro-Wilk W test) before analysis. Unequal sample sizes were corrected for by this software program, and adjusted *F*-statistics were reported. Plasma and brain ChE activity and response to the lectin PHA did not meet the requirements and were therefore $\log_{10}$ transformed before analysis. To determine if fasting time (1 hour versus overnight) had an effect on brain ChE response, an analysis of covariance (ANCOVA) was conducted with time of fasting time as a covariate. Cholinesterase activities were similar in birds fasted for 1 hour to those fasted overnight before administration of the OP ($F_{1,38}$ = 1.12, p = 0.2969); therefore, these data were pooled for subsequent analysis. A regression analysis correcting for body mass was conducted to determine the relationship between increasing dosages of azinphos-methyl and plasma and brain ChE activity. To determine if a difference existed in plasma and brain ChE response to azinphos-methyl exposure in birds exposed to low, medium, and high levels of *p,p′*-DDE, ANCOVA was conducted with body mass as a covariate. If a difference existed, Tukey's Honestly Significantly Different (HSD) test was used for multiple comparisons. Because body mass was not correlated with the remaining parameters tested (PHA response or percent hematocrit and leucocrit), a least-squares analysis of variance was conducted to see if a difference existed among the treatments. If a difference existed, this was followed by Tukey's HSD for multiple comparisons. Because hematocrit and leucocrit values were converted to percentages, data were arc-sine transformed before analysis. Further statistical analyses were also conducted to determine the individual pesticide effects on the hematologic and immune function parameters (*i.e.*, birds previously exposed to *p,p′*-DDE 1 year before the start of the experiment and those exposed to azinphos-methyl).

Results

Confirmation of Pesticide Contaminated Egg Food Preparation and Liver Residues in p,p′*-DDE Pre-Exposed Zebra Finches*

Measured concentrations of *p,p′*-DDE in treated egg food were within an average of 15% of the target doses (Table 2), thus confirming correct preparation of *p,p′*-DDE–contaminated egg food. Male zebra finches exposed to *p,p′*-DDE 1 year previously continued to have increased levels in liver compared with nonexposed birds; however, all levels were lower than those livers tested immediately after exposure (Table 3). OC contaminants in livers of males birds after 4 weeks of exposure to *p,p′*-DDE at 19 mg/kg, 34 mg/kg, and 60 mg/kg were generally much higher and are summarized in Table 3. In addition to *p,p′*-DDE, some birds also had traces of γ-hexachlorocyclohexane residues in their liver. All other OC compounds tested for were nondetectable in either food or liver.

Experiment 1. Dose-response of plasma and brain ChE activity to azinphos-methyl. Abnormal behavior (*i.e.*, tremors, convulsions, lethargy) was not observed in any birds throughout this study, including those observed for 1 week after dosing with azinphos-methyl. One bird died within 5 seconds after dosing (most likely because trauma) in the 1.5-mg/kg dosage group and therefore was not analyzed for ChE activity. Plasma and brain ChE activity decreased linearly with increasing dosage of azinphos-methyl (plasma ChE activity: $F_{1,51}$ = 69.8, $p < 0.0001$; brain ChE activity: $F_{1,38}$ = 105.9, $p < 0.0001$; Fig. 1). From those results we determined that 20% to 25% brain ChE inhibition would occur at approximately 18.4 mg/kg azinphos-methyl.

Table 2. Concentrations of *p,p′*-DDE (mg/kg, wet weight) in contaminated egg food used to dose zebra finches[a]

Sample (mg/kg egg food)	Lipid (%)	Moisture (%)	*p,p′*-DDE
19	16.7	72	16.4
19	17.7	54	17.2
34	15.2	46	36.8
34	14.7	46	39.4
60	15.2	55	63.8
60	15.5	63	56.8

[a] No other OC compounds tested for were detected.
OC = Organochlorine.

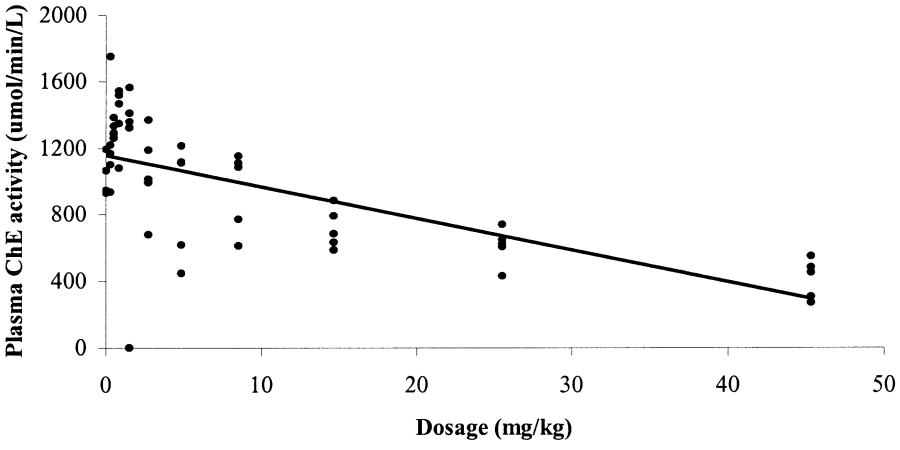
Table 3. OC concentrations (mg/kg wet weight) in male zebra finch livers exposed to *p,p′*-DDE at 19, 34, and 60 mg/kg[a]

Sample	*p,p′*-DDE Dosage (mg/kg)	Lipid (%)	Moisture (%)[b]	*p,p′*-DDE	γ-HCH
1	Corn oil	11.8	–	3.77 (31.9)	ND
2	Corn oil	1.74	–	1.25 (71.8)	ND
3	Corn oil	2.56	–	4.14 (161)	0.003 (0.117)
4	19	3.72	–	8.12 (218)	0.57 (15.3)
5	19	5.77	–	7.59 (132)	0.03 (0.520)
6	34	7.98	–	159 (1990)	6.16 (77.2)
7	34	1.87	–	36.2 (1990)	0.56 (29.9)
8	60	8.33	–	60.7 (728)	1.80 (21.6)
9	60	8.11	–	126 (1550)	1.00 (12.3)

[a] Lipid normalized values are presented in parentheses. In addition, livers from controls (exposed to plain corn oil) were sent in for comparison. All birds were additionally exposed to *p,p′*-DDE (34 mg/kg) approximately 1 year before tissue collection. No other OC compounds tested for were detected.
[b] This was not determined for these samples because of insufficient sample size.
ND = not determined, OC = Organochlorine.

Experiment 2. Effects of *p,p′*-DDE and azinphos-methyl on ChE activity and general health. The PHA test was conducted after 3 weeks of exposure to plain corn oil or *p,p′*-DDE. There was no response to the lectin in the five treatment groups (range 0.31 to 1.23 mm, $F_{4,53}$ = 2.50, p = 0.0535; Table 4). Birds exposed 1 year previously to *p,p′*-DDE (mean 0.65 ± 0.03 mm, n = 38) had a significantly higher response to the plant lectin compared with those not previously exposed (mean 0.52 ± 0.04 mm, n = 20) ($F_{1,56}$ = 7.86, p = 0.0069).

After 4 weeks of exposure to oil or DDE, birds were orally dosed with the OP azinphos-methyl. Three hours after that dosage, hematocrit values were higher in the control birds (corn oil plus corn oil) than those exposed to the corn oil plus azinphos-methyl (range 40.3% to 63.5%, $F_{5,51}$ = 3.41, p = 0.0098; Table 5). In addition, significantly lower hematocrit values were observed in birds exposed 1 year previously



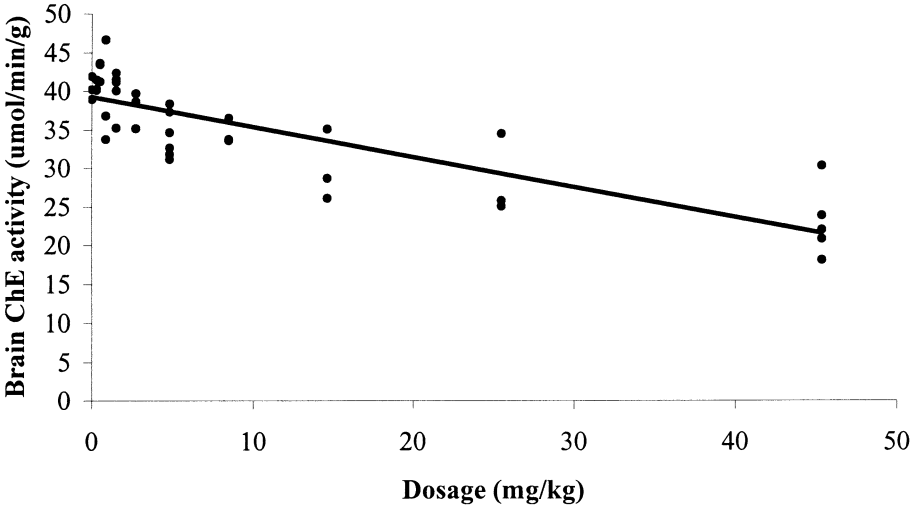


Fig. 1. Plasma and brain ChE activity in adult male zebra finches orally dosed with the OP azinphos-methyl. Both plasma ($p < 0.0001$, adjusted $R^2 = 0.57$) and brain ($p < 0.0001$, adjusted $R^2 = 0.72$) ChE activity decreased linearly with increasing dosage of azinphos-methyl. ChE = Cholinesterase; OP = organophosphate

to *p,p′*-DDE (mean: 53.6 ± 0.8, n = 37) compared with those not previously exposed (mean: 56.8 ± 1.0, n = 20 ($F_{1,55}$ = 6.40, $p = 0.0143$). Current azinphos-methyl exposure also caused lower hematocrit values (mean 54.1 ± 0.7, n = 47) compared with controls (57.7 ± 1.55, n = 10) ($F_{1,55} = 5.12$, $p = 0.0276$). Percent leucocrit was higher in birds exposed to azinphos-methyl (0.87 ± 0.06, n = 47) compared with those not exposed (0.55 ± 0.14, n = 10) ($F_{1,55} = 4.60$, $p = 0.0363$). There was no difference in percent leucocrit amongst the combination of treatments ($F_{5,51} = 1.76$, $p = 0.1384$; Table 5) and those exposed to *p,p′*-DDE 1 year previously (mean *p,p′*-DDE 0.86 ± 0.07, n = 37; mean corn oil: 0.74 ± 0.10, n = 20; $F_{1,55} = 0.82$, $p = 0.3679$). Both plasma ($F_{5,48} = 22.2$, $p < 0.0001$) and brain ($F_{5,51} = 18.1$, $p < 0.0001$) ChE activities were inhibited after exposure to azinphos-methyl; however, there was no difference in the extent of inhibition in birds exposed to low, medium, and high dosages of *p,p′*-DDE (Figure 2).

Discussion

The main results of our study were as follows: (1) azinphos-methyl resulted in a dose-dependent inhibition of plasma and brain ChE activity; (2) *p,p′*-DDE in combination with azinphos-methyl did not change azinphos-methyl inhibition of ChE activity; and (3) there were suggestions of immunostimulation in birds dosed 1 year previously to *p,p′*-DDE and of anemia when *p,p′*-DDE was combined with azinphos-methyl; how-

Table 4. Comparison of PHA skin test response in adult male zebra finches treated with plain corn oil or *p,p′*-DDE at low, medium, or high concentrations (19, 34, and 60 mg/kg)[a]

Dosage Group[b]	n	PHA Response (mm)
Corn oil	20	0.52 ± 0.04
Corn oil (DDE)	9	0.77 ± 0.06
19 mg/kg *p,p′*-DDE (DDE)	10	0.63 ± 0.06
34 mg/kg *p,p′*-DDE (DDE)	10	0.60 ± 0.06
60 mg/kg *p,p′*-DDE (DDE)	9	0.61 ± 0.06
p value		0.0535

[a] Values are expressed as the least squares mean ± standard error. Statistical analysis was conducted using a least squared analysis of variance (ANOVA).
[b] Those with DDE in parentheses indicate birds that were previously exposed to *p,p′*-DDE (34 mg/kg) approximately 1 year previously. ANOVA = Analysis of variance, PHA = phytohemagglutinin.

ever, there was no dose-response for these parameters in birds subsequently dosed with *p,p′*-DDE.

Validation of Methodology and Dosing

Because many birds used in this experiment were dosed with *p,p′*-DDE 1 year previously, it was important to determine the extent of the contaminant remaining before the start of the current experiment. Approximately 1 year after dosing, zebra finches generally had increased *p,p′*-DDE residues in liver. Few data are available for liver DDE residues remaining after long-term dosing in birds. However, in the lower mainland of British Columbia, American robins residing in habitat with no previous DDT usage had 0.3 to 3.2 μg/g DDE in eggs (Gill *et al.* 2003). Assuming a similar egg-to-liver ratio as that reported in herring gulls (*Larus argentatus*) (Braune and Norstrom 1989), robins with DDE in eggs ranging from 0.3 to 3.2 μg/g DDE would have liver residues of 0.34 to 3.64 μg/g DDE. Thus, DDE concentrations were comparable with those in robins from noncontaminated areas. Zebra finch livers also had low levels of γ-hexachlorocyclohexane, a commonly used seed treatment (although the actual source of this contaminant to birds in our study was not confirmed). Because all birds, including controls, received the same seed, we would not expect any significant effects from this pesticide.

Effects of Azinphos-Methyl on Cholinesterase Activity

Zebra finches exposed to azinphos-methyl exhibited a dose-related increase in brain and plasma ChE inhibition. This is a typical response seen in avian species after exposure to OPs such as fenitrothion (Holmes and Boag 1990) and quinalphos (Anam and Maitra 1995). Maximum brain ChE inhibition at our highest dose of 45.3 mg/kg resulted in 42.9% inhibition of the enzyme. Brain ChE inhibition of 50% is an indicator of OP exposure resulting in death (Ludke *et al.* 1975; Busby *et al.* 1981). However, birds can have inhibition >50% and still survive. Our birds were approaching brain ChE inhibition of 50% and did not behave abnormally or die. Zebra finches exposed to fenitrothion survived after dosages that resulted in

Table 5. Percent hematocrit and leucocrit in male zebra finches exposed to corn oil or *p,p′*-DDE at low, medium and high doses and with the OC azinphos-methyl[a]

Dosage group[b]	Hematocrit (%)	Leucocrit (%)
Corn oil + corn oil	57.7 ± 1.4 (10)[1]	0.55 ± 0.14 (10)
Corn oil + azinphos-methyl	55.8 ± 1.4 (10)[1,2]	0.94 ± 0.14 (10)
Corn oil + azinphos-methyl (DDE)	50.7 ± 1.5 (9)[2]	0.70 ± 0.15 (8)
19 mg/kg *p,p′*-DDE + azinphos-methyl (DDE)	55.6 ± 1.4 (10)[1,2]	0.73 ± 0.14 (10)
34 mg/kg *p,p′*-DDE + azinphos-methyl (DDE)	52.1 ± 1.5 (9)[1,2]	1.02 ± 0.14 (10)
60 mg/kg *p,p′*-DDE + azinphos-methyl (DDE)	55.6 ± 1.5 (9)[1,2]	0.96 ± 0.14 (9)
p value	0.0098	0.1384

[a] Values are expressed as least squared mean ± SE with sample size in parentheses. Statistical analysis was conducted using a least-squares analysis of variance (ANOVA) followed by Tukey's HSD for multiple comparisons. Mean values followed by the same superscript numbers indicate values that are not significantly different. Because hematocrit and leucocrit values were converted to percentages, data was arc sine transformed before analysis.
[b] Those with DDE in parentheses indicate birds that were previously exposed to *p,p′*-DDE (34 mg/kg) approximately 1 year previously. ANOVA = Analysis of variance, HDS= Honesty Significantly Different.

>50% brain ChE inhibition (Holmes and Boag 1990). Thus, zebra finches appear to be more resistant to azinphos-methyl exposure compared with other songbird species. Our birds were administered the pesticide at dosages as high as 45.3 mg/kg with no mortality. Much lower LD_{50}s have been measured in other songbird species, *e.g.*, red-winged blackbird (*Agelaius phoeniceus*), 8.5 mg/kg and European starlings (*Sturnus vulgaris*), 27 mg/kg (Smith 1987).

Effects of p,p′*-DDE and Azinphos-Methyl on Cholinesterase Activity*

Earlier studies with both mice and rats showed that OC compounds increased ChE enzyme activity and thus decreased the response to subsequent OP exposure (Ball *et al.* 1954; Triolo and Coon 1966; Menzer 1970). Rats given a single oral dose of aldrin, chlordane, or lindane were protected 4 days later against subsequent parathion exposure; *i.e.*, plasma ChE activity was less inhibited with pre-exposure to the chlorinated hydrocarbons than with no pre-exposure (Ball *et al.* 1954). Isomers of DDT have been reported to accelerate several detoxification processes in rat liver microsomes including the hydrolysis of paraoxon (the anti-ChE form of the OP parathion) and dealkylation of some OPs (Crevier *et al.* 1954). Lower mortality is also seen in mice pretreated with chlorinated hydrocarbons followed by OP exposure than those exposed to the OP alone (Triolo and Coon 1966).

We found no effect of pretreatment with DDE on azinphos-methyl inhibition of ChE in the brain and plasma of zebra finches. In contrast to mammals, avian studies have shown that DDE exposure does not decrease the effects of subsequent OP

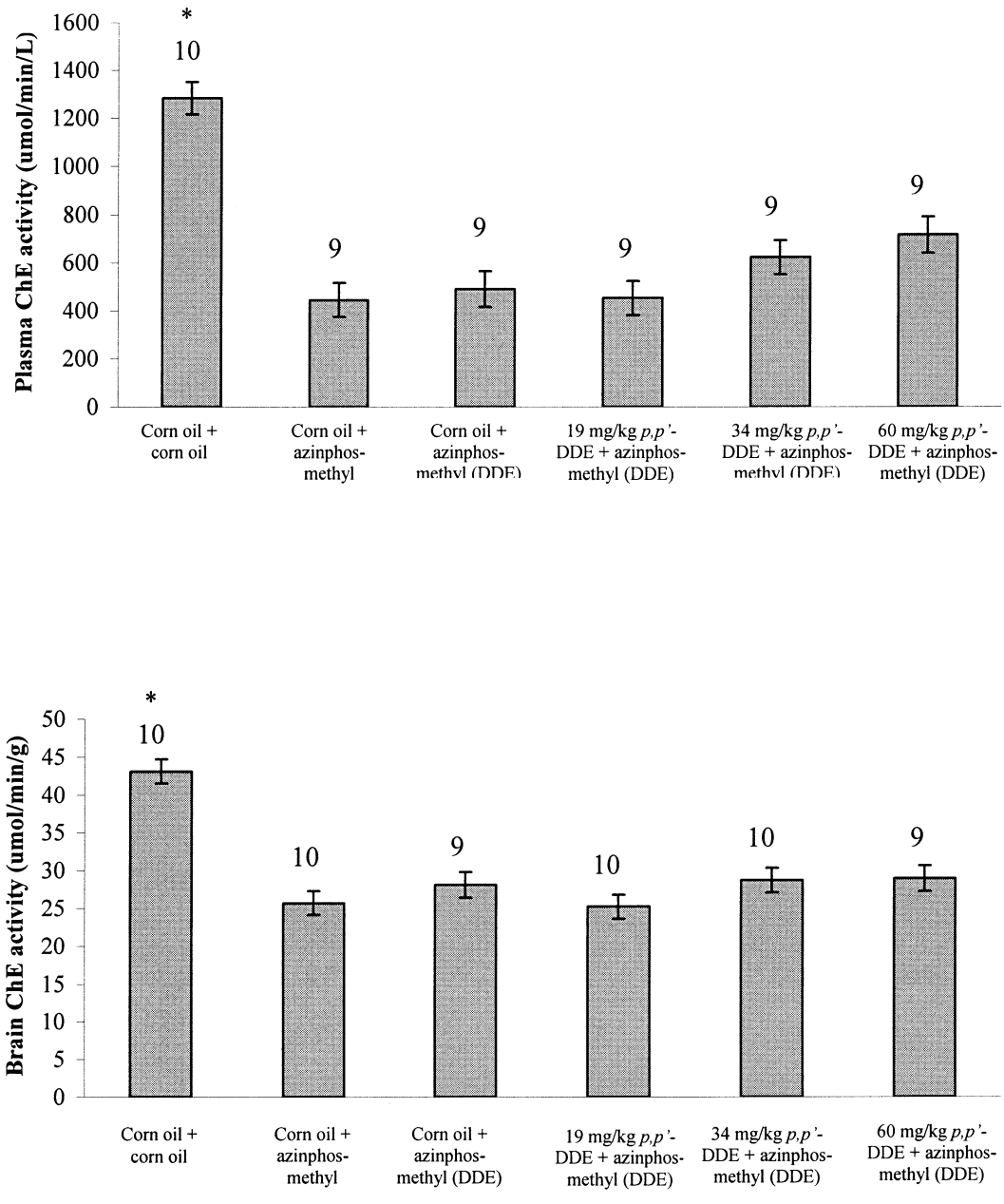


Fig. 2. Plasma and ChE activity in adult male zebra finches exposed to plain corn oil or *p,p′*-DDE and the OP azinphos-methyl. Those with DDE in parentheses indicate birds that were previously exposed to *p,p′*-DDE (34 mg/kg) approximately 1 year previously. Results shown are the least-squares mean ± standard error with the sample size presented above each bar. Statistical analyses were conducted using a least-squared ANCOVA with weight of the bird as a covariate. This was followed by Tukey's HSD for multiple comparisons. Bars with asterisks are statistically different from those with no asterisks ($p < 0.0001$ for both plasma and brain ChE activity). ANCOVA = Analysis of covariance; ChE = cholinesterase; HSD = Honestly Significantly Different; OP = organophosphate

exposure but that it may actually increase the toxicity of OPs. Coturnix quail exposed to DDE showed a dose-related increase in plasma ChE activity (Dieter 1974; Ludke 1977). However, Ludke (1977) showed that this increase in plasma ChE activity did not minimize the effects of subsequent exposure to the OP parathion as was reported in the laboratory mammals discussed previously. Those Coturnix quail without pretreatment to DDE displayed relatively lower plasma ChE inhibition than did DDE-pretreated birds when they were subsequently challenged with parathion, which suggests that any protection was offset by a net difference resulting from some other (unidentified) pretreatment effect. Azinphos-methyl does not require activation to another chemical form to be a potent ChE inhibitor; therefore, the potential role of DDE as, for example, a cytochrome P-450 inducer—as reported for the fungicide prochloraz (Johnston *et al.* 1994)—would not be a factor here.

Direct Effects of p,p′*-DDE and Azinphos-Methyl on General Health*

There has been an increased interest in the effects of environmental toxins on the immune system (Dethloff and Bailey 1998; Smits *et al.* 1996; Smits and Williams 1999). The PHA immune function test is an increasingly common assay of cell-mediated immune function. In our study, the PHA response was higher in birds with previous exposure to *p,p′*-DDE (approximately 1 year before the start of the experiment), which suggests immunostimulation in *p,p′*-DDE exposed birds. This immunostimulation was further supported by the increase in leucocrit values in birds exposed to azinphos-methyl. These findings are consistent with results obtained in a field study in which tree swallows exposed to orchard pesticides, including azinphos-methyl and *p,p′*-DDE, exhibited immunostimulation (Bishop *et al.* 1998). The study, conducted by Bishop *et al.* (1998), was the first field study conducted to examine immune parameters in wild birds exposed to pesticides. Our study conducted in a laboratory setting further supports this suggestion of immunostimulation in songbirds exposed to orchard pesticides. This is in contrast to previous studies on humans who were occupationally exposed to pesticides and captive animals dosed with pesticides; both studies of humans and captive animals showed the most common response of the immune system was suppression (Vos *et al.* 1989; Dean and Murray 1991; Pruett 1994).

Hematocrit values were lower in birds exposed to corn oil plus azinphos-methyl (with previous exposure to *p,p′*-DDE approximately 1 year previously) than the control birds (corn oil plus corn oil). However, other groups with pesticide exposure did not show any difference in hematocrit response; therefore, it is likely that this may be a spurious relationship. However, when looking at individual pesticide effects, birds exposed to *p,p′*-DDE previously (approximately 1 year before the start of the experiment) and those exposed to azinphosmethyl had lower hematocrit values than those with no pesticide exposure, which suggests an anemic response to pesticides. Bishop *et al.* (1998) found the same trend in tree swallows exposed to orchard pesticides, *i.e.*, exposed birds also had lower hematocrit levels.

Conclusions

In conclusion, although zebra finches experienced ChE inhibition after exposure to azinphos-methyl, they appeared to be less sensitive to OP exposure compared with other songbirds. This lower inhibition is important if zebra finches are to be used as a model species in toxicologic studies. Although *p,p′*-DDE did not appear to affect the degree of ChE inhibition after subsequent azinphos-methyl exposure, it did appear to result in stimulation of the immune system and possible anemia. Further studies are warranted to determine pesticide effects on the immune system to further understand and interpret these results.

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