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Monitoring Exposure of Passerines to Acephate, Dicrotophos, and Malathion Using Cholinesterase Reactivation

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The Mississippi Alluvial Plain physiographic region of eastern Arkansas provides important avian breeding and migratory stopover habitat. Within this region, agriculture is a dominant land use. Primary crops produced include cotton, soybean, and rice (USDA 2003a), and organophosphorus (OP) pesticides are used extensively to control crop damage from insects. The most frequently and abundantly applied insecticides on Arkansas cotton fields in 2001 were the OP compounds acephate (O,S-dimethyl acetylphosphoramidothioate) (9.13×10^4 kg applied), dicrotophos (dimethyl phosphate of 3-hydroxy N,N-dimethyl-cis-crotonamide) (1.36×10^4 kg), and malathion (O,O-dimethyl phosphorodithioate) (6.46×10^5 kg) (USDA 2003b).

Avian exposure to OP compounds may be more prevalent in species with affinity for field edges and can occur by direct consumption of insects or seeds containing residues, pesticide drift, and contact with OP-coated vegetation (Gard and Hooper 1995). Exposure may result in effects ranging from physiological to behavioral (Grue et al. 1991; Gard and Hooper 1995). Reductions in body mass and fat have been observed for passerines after dietary exposure to some OP compounds (Grue 1982; Rattner and Grue 1990).

Quantifying brain or plasma cholinesterase (ChE) enzyme activity is a widely used method to monitor avian exposure to OP pesticides (McInnes et al. 1996; Parsons et al. 2000). Examination of chemical reactivation of ChE enzyme is an additional monitoring tool used to assess OP exposure to avifauna (Wilson et al. 1992) and has been used successfully in monitoring red-tailed hawk (*Buteo jamaicensis*) exposure to OPs (Hooper et al. 1989) and chlorpyrifos exposure to nestling red-winged blackbirds (*Agelaius phoeniceus*) (McInnes et al. 1996).

The objectives of this study were to: 1) monitor exposure of passerines to the OP pesticides acephate, dicrotophos, and malathion applied to cotton fields under typical application regimes by measuring plasma total cholinesterase (ChE) reactivations and 2) compare body mass and fat of passerines having plasma ChE that reactivated to those with no plasma ChE reactivation.

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MATERIALS AND METHODS

Three sampling locations were located at the edge of cotton production fields in Poinsett Co., AR adjacent to 2 < 30 m wide drainage ditches containing herbaceous and woody vegetation (F1 and F2) and a small area of drained wetland containing shrub and saplings interspersed with mature cypress trees (F3). A fourth site was located at Wapanocca National Wildlife Refuge (WNWR). Acephate (0.252 and 0.336 kg active ingredient [a.i.]/ha), dicrotophos (0.280 kg a.i./ha), and malathion (0.437 kg a.i./ha) were applied to fields adjacent to sampling locations by farm operators. Farm managers made decisions on choice of compound and timing of application for insect control. Passerines were captured at field edges with mist nets (2.6 × 12 m, 36 mm mesh) between 9 May 2001 and 5 August 2002. Body mass and fat were recorded for all passerines monitored during this study. Changes in mass and body fat have been measured as a response in passerines to OP exposure in dosing and dietary exposure experiments (Grue 1982; Rattner and Grue 1990). Body mass was measured with a 60 g (± 0.1 g) or 100 g (± 0.5 g) spring scale. Fat deposits were scored as an indicator of individual condition through visual estimation by a single observer (JDM). This method is an acceptable index of overall body fat if inter-observer variation is controlled (Krementz and Pendleton 1990). Fat classes were based on the amount of fat deposited in the furcular cavity following Grue (1982) such that: 1 = absence of fat, 2 = partial fat deposition between clavicles, 3 = concave sheet of fat covering 100% of furcular cavity, and 4 = fat filling furcular cavity and covering clavicles.

Blood was collected from the jugular vein with heparinized 1 mL syringes, transferred to heparinized microcentrifuge tubes, and immediately placed on ice. Blood samples were centrifuged at 3700 × g for 7 min at 4°C and plasma was retrieved and stored at –70°C. Total plasma cholinesterase (ChE) and acetylcholinesterase (AChE) activities were measured colorimetrically using the methods of Ellman et al. (1961) and modifications thereof (Gard and Hooper 1993), using a 96 well microplate spectrophotometer in kinetics mode at 25°C. Absorbance readings were taken every 15 sec for 2 min at $\lambda = 412$ nm. The two cholinesterases typically observed in avian plasma (AChE and butyrylcholinesterase [BChE]; Thompson 1991) were differentiated using the BChE-specific inhibitor tetraisopropyl pyrophosphoramide (Iso-OMPA) with BChE being the difference between measured total ChE and AChE activities. Differentiating as such can improve the sensitivity of this biomarker for detecting enzyme inhibition from OP exposure (Fairbrother et al. 1991). Acetylthiocholine iodide (AThCh) was used as a substrate for the cholinesterase enzymes, and enzyme activity was expressed as μmoles of AThCh hydrolyzed/min/mL plasma. The following reagent volumes and final concentrations (FC) were added to appropriate microplate wells for the assay: 1) 150 μL of 0.05 *M* Tris buffer at pH 8.0, 2) 20 μL of 5',5'-dithiobis-(2-nitrobenzoic acid) (DTNB) [FC = 3.23×10^{-4} *M*], 3) 30 μL aliquot of diluted (10- or 20-fold) and vortexed plasma to sample wells and 30 μL of pH 8.0 Tris buffer to blank wells, 4) 20 μL of Iso-OMPA [FC = 1.10×10^{-4} *M*] to selectively inhibited BChE wells and 20 μL of pH 8.0 Tris

buffer to un-inhibited wells, and 5) 30 µL of AThCh [FC = 1.0×10^{-3} *M*] to all wells. The final assay volume was 250 µL/microplate well.

A reactivation assay was conducted in which ChE bound to OP compounds was reactivated by spiking sample aliquots with pyridine-2-aldoxime methochloride (2-PAM) (3.23×10^{-4} *M* final concentration in each well) and incubating at 25°C (Martin et al. 1981; Hooper et al. 1989). Indication of ChE enzyme reactivation in samples followed criteria described in Parsons et al. (2000). Specifically, enzyme reactivation was considered to occur when aliquots of samples spiked with 2-PAM and incubated for 30 min at 25°C had an increase of ChE activity >5% compared to samples incubated without 2-PAM and when mean activities of spiked and unspiked aliquots were statistically different (*t*-test, $\alpha = 0.05$) (PROC TTEST, SAS Institute Inc., 1989). The reactivation assay was used because it does not require comparison of ChE activity data from unexposed reference populations, detecting OP exposure can occur without knowledge of absolute ChE activity of samples (Hooper et al. 1989), and potential sources of variation in ChE enzyme activity among samples were controlled (McInnes et al. 1996) facilitating multi-species monitoring. All chemicals were obtained from Sigma Chemical Company, St. Louis, MO, USA.

Standards and blanks were run simultaneous to samples for each plate and each sample was run in triplicate. Standards consisted of horse serum and functioned to ensure that reagent concentrations and pipetting were accurate while blank wells contained all reagents except enzyme.

RESULTS AND DISCUSSION

We analyzed 62 plasma samples from 8 passerine species including: American robin (species code = AMRO), *Turdus migratorius*; blue jay (BLJA), *Cyanocitta cristata*; brown thrasher (BRTH), *Toxostoma rufum*; brown-headed cowbird (BHCO), *Molothrus ater*; dickcissel (DICK), *Spiza americana*; gray catbird (GRCA), *Dumetella carolinensis*; red-winged blackbird (RWBL); and yellow-breasted chat (YBCH), *Icteria virens* (Table 1). Reactivation of ChE enzyme was detected for 5.9% (3/51) of passerines sampled from fields after OP applications (Table 1 and 2). This was less than the percentage of reactivations observed for passerines using golf courses treated with OP's and carbamates (20.5%) (Rainwater et al. 1995) and nestling RWBL from OP treated fields (34.1%) (McInnes et al. 1996). This result was unexpected considering the frequency of OP applications and the proximity of sampled birds (and their probable breeding territories) to treated fields. The 3 ChE reactivations occurred in birds that were sampled at sites within 4 days of an OP application (Table 2). These species (AMRO, DICK, and RWBL) were frequently observed foraging and/or defending territories within cotton fields, whereas other species were only observed at field edges.

Zinkl et al. (1980) reported that 3 days after a 0.57 kg a.i./ha acephate treatment of forest plots, 93% ($n = 14$) of passerines had depressed brain ChE levels (i.e.,

Table 1. Total plasma cholinesterase (ChE) activity, acetylcholinesterase (AChE) activity, and ChE reactivations (ChE-R) of passerines sampled after organophosphorous (OP) pesticide applications at study sites in northeastern Arkansas during 2001 and 2002.

Species[a]	Site	*n*	Days Post Application[b]	Mean ± s.d. ChE Activity[c]	Mean ± s.d. AChE Activity[c]	No. of ChE-R
AMRO	F3	1	2M, 4M	1.46	0.23	0
	F2	1	8M, 12D, 23A	1.99	0.31	0
	F1	6	5M, 24D	1.17 ± 0.31	0.26 ± 0.15	1
	F2	3	--	1.19 ± 0.28	0.26 ± 0.08	0
BHCO	F3	1	1A	1.51	0.20	0
	F3	1	<1M, 23A	2.60	0.11	0
	WNWR	5	--	2.14 ± 0.99	0.10 ± 0.05	0
BLJA	F3	1	1A	1.08	0.51	0
	F3	1	4A	0.90	0.52	0
BRTH	F3	1	4A	0.36	0.36	0
	F2	1	<1M, 1A, 18D	0.78	0.77	0
	F1	1	--	0.99	0.66	0
DICK	F3	4	1A	1.53 ± 0.56	1.36 ± 0.48	0
	F3	4	4A	2.44 ± 0.51	2.16 ± 0.48	1
	F3	1	3M, 13A	1.56	1.47	0
	F3	3	3M	2.22 ± 0.21	1.93 ± 0.18	0
	F3	4	6M	1.86 ± 0.51	1.64 ± 0.43	0
GRCA	F3	2	1A	1.23 ± 0.26	0.35 ± 0.13	0
RWBL	F3	1	4A	3.60	0.17	0
	F1	1	<1D, 6D, 10A	0.14	0.03	1
	F3	9	<1M, 23A	2.84 ± 0.76	0.15 ± 0.06	0
	F3	4	3M	3.19 ± 0.54	0.17 ± 0.04	0
YBCH	F3	2	<1M, 23A	1.93 ± 0.01	0.07 ± 0.01	0
	F3	1	6M	1.93	0.10	0
	F3	1	2M, 4M	1.76	0.07	0
	F3	2	--	1.12 ± 0.12	0.08 ± 0.0	0

[a] American robin (AMRO), brown-headed cowbird (BHCO), blue jay (BLJA), brown thrasher (BRTH), dickcissel (DICK), gray catbird (GRCA), red-winged blackbird (RWBL), and yellow-breasted chat (YBCH).

[b] Number of days since application of: acephate (A) [Site F1 & F2 = 0.336 kg active ingredient (a.i.)/ha, Site F3 = 0.252 kg a.i./ha]; dicrotophos (D) [0.280 kg a.i./ha], and malathion (M) [0.437 kg a.i./ha]; -- = no known OP applications prior to avian sampling.

[c] µmoles of acetylthiocholine (AThCh) hydrolyzed/min/ml plasma.

control – 2 s.d.). In our study, acephate was applied at 0.252 and 0.336 kg a.i./ha with blood samples collected from 15 passerines 1 and 4 days after application and reactivation was observed for only one individual (Table 1). This pattern suggests that small non-agricultural and drainage ditch habitats may provide some measure of refuge to passerines from agrochemical applications; however, care

Table 2. Total plasma cholinesterase (ChE) activity (mean ± s.d.) from passerines sampled after organophosphorous (OP) pesticide applications that had a significant ChE reactivation.

Species[a]	Site	ChE Activity[b] no 2-PAM	ChE Activity[b] with 2-PAM	Increase in ChE (%)	*t*	*P*
AMRO	F1	1.65 ± 0.042	1.82 ± 0.031	9.5	4.98	0.016
DICK	F3	1.77 ± 0.009	1.90 ± 0.040	7.0	4.46	0.021
RWBL	F1	0.14 ± 0.007	0.27 ± 0.004	46.8	29.29	<0.0001

[a] American robin (AMRO), dickcissel (DICK), and red-winged blackbird (RWBL). For each individual, means from sample aliquots with (n = 3) and without pyridine-2-aldoxime methochloride (2-PAM) (n = 3) are presented.

[b] µmoles of acetylthiocholine (AThCh) hydrolyzed/min/ml plasma.

should be taken in comparing these data with those reported by Zinkl et al. (1980) because different methods were used for assessing OP exposure (i.e., ChE reactivation vs. ChE depression). In another study, Rainwater et al. (1995) did not detect OP associated ChE reactivations in 2 passerine plasma samples collected from animals captured on a golf course 2 days after a 4.20 kg a.i./ha application of acephate.

Avian samples were examined following pesticide application regimes that varied with respect to pesticide combination, timing of application, and concentration of active ingredient (Table 1). Although this made it difficult to identify specific compounds responsible for inhibited ChE enzyme, it presented a realistic exposure scenario of mixtures and multiple applications. A possible inhibition of RWBL ChE was likely attributable to dicrotophos and acephate applied 6 and 10 days prior to sampling (Table 2); a 46.8% increase in ChE activity was observed after incubation in the presence of 2-PAM during the reactivation assay. Reactivations of up to 71.4% have been reported for common grackle, *Quiscalus quiscula*, without overt signs of poisoning (Rainwater et al. 1995). All birds captured and sampled in this study, including those that had ChE reactivation, did not exhibit signs of poisoning.

Differences in body mass and fat score between birds exhibiting ChE reactivation and those without reactivation were not compared statistically due to small sample size (Table 3). Body mass of an AMRO and RWBL with reactivated ChE, however, was within the 95% CI observed for mass of AMRO and RWBL without reactivation of ChE enzyme (Table 3). This agrees with Grue (1982) and Zinkl et al. (1981) who found no difference in body mass changes in control and surviving birds exposed to dicrotophos and acephate via diet. However, birds that died in both studies had significant weight reductions due to reduced food consumption (Zinkl et al. 1981; Grue 1982). Fat score was similar between the two groups for AMRO, DICK, and RWBL (i.e., birds with reactivation were within the 95% CI for those not exhibiting ChE reactivations) (Table 3). Grue (1982) reported a difference in percentage of birds within fat score classifications between control and exposed birds during late summer, but not during spring

Table 3. Mass and fat score for 3 passerines that exhibited ChE reactivation and those without ChE reactivation.

Response	Species[a]	ChE Reactivation[c]	*n*	No ChE Reactivation[d]	*n*
Mass (g)	DICK[b]	32.0	1	27.8 (26.9-28.8)	9
	AMRO	69.0	1	69.0 (64.1-73.9)	7
	RWBL[b]	59.3	1	56.9 (51.9-61.9)	4
Fat Score	DICK[b]	2.0	1	2.0 (1.7-2.3)	9
	AMRO	2.0	1	2.1 (1.9-2.4)	7
	RWBL[b]	1.0	1	1.3 (0.8-1.7)	4

[a] American robin (AMRO), dickcissel (DICK), and red-winged blackbird (RWBL).
[b] Males only; because of sexual dimorphism females were excluded.
[c] Actual mass and fat score values given.
[d] Mean (95% Confidence Interval).

experiments. Indirect effects of pesticide applications attributable to reductions in food resources may be an important indirect factor in reduced avian fat scores, but variation in abundance of invertebrate prey in application areas was not examined in this study.

Passerine species that commonly forage or nest within agricultural fields (e.g., DICK and RWBL) may be at greater risk of exposure to OP pesticides than those remaining in non-agricultural edge habitats as suggested by the pattern of plasma ChE reactivations among species. Non-agricultural areas adjacent to crop fields may provide some protection from exposure, but the extent of this protection needs to be examined further. In addition, multiple applications of different compounds that can vary in mode of toxic action are used for pest control on cotton fields. Thus, future research on agricultural pesticide impacts to passerines should focus on effects of chemical mixtures and repeated exposures.

We also recommend that future work place an effort on maximizing sample sizes since percent reactivations in this system were just over 5.0% and close to what might be expected from chance alone when using a significance level of 0.05.

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