2013. Proceedings of the Indiana Academy of Science 121(2):133–142

SCREENING OF INSECTICIDES IN BATS FROM INDIANA

Ronny R. Eidels and **John O. Whitaker, Jr.**: Center for North American Bat Research and Conservation, Department of Biology, Indiana State University, Terre Haute, IN

Michael J. Lydy: Center for Fisheries, Aquaculture and Aquatic Sciences and Department of Zoology, Southern Illinois University, Carbondale, IL

Daniel W. Sparks: U. S. Fish & Wildlife Service, Bloomington Field Office, Bloomington, IN

ABSTRACT. This study identified insecticides that were detected in bats obtained from Indiana's Lake Michigan watershed. Forty bats collected from Lake, Porter and LaPorte Counties, Indiana, were analyzed for pyrethroid, organochlorine, organophosphate and carbamate insecticides. Additionally, brain cholinesterase activity of 332 bats from throughout Indiana was measured and cholinesterase reactivation tests were performed. Organochlorine pesticides (dieldrin, DDT, DDE, DDD and heptachlor epoxide) were detected in 97.5% of the tested bats; organophosphate compounds (primarily diazinon) were detected in 30%; pyrethroids in 12.5% and carbamates in 2.5% of the bats, respectively. Cholinesterase determination and reactivation tests yielded both false negative and false positive errors, which indicate that reactivation methods are not suitable for analyzing tissues from animals that are not recently dead. These results are among the first reported detections of pyrethroids and carbamates in bat tissues.

Keywords: Insecticides, bats, exposure, sentinels

INTRODUCTION

Bats are the primary predator of night flying insects including many agricultural pests (Lee & McCracken 2005; Whitaker 1995; Whitaker & Hamilton 1998). Whitaker (1995) estimated that a typical Midwestern big brown bat (*Eptesicus fuscus*) colony of 150 bats may consume in a season 600,000 spotted cucumber beetles (*Diabrotica undecimpunctata, Chrysomelidae*), 194,000 scarab beetles (*Scarabaeidae*), 158,000 leafhoppers (*Cicadellidae*) and 335,000 stinkbugs (*Pentatomidae*). All of these are significant pest species. Other bat species feed on moths such as the Turnip moth (*Noctuidae*), the larva of which (cutworm) is an important garden pest (Whitaker & Hamilton 1998). As such, bats are likely to play an important role in reducing damage to crops. Bat populations worldwide are declining (Kunz & Fenton 2003). In Indiana, several of the bat species are also declining (Whitaker et al. 2002). This is of particular concern because Indiana has the largest hibernating populations of the federally endangered Indiana bat (*Myotis sodalis*). During the winter of 2007, 50.8% of the total world population of this bat species hibernated in Indiana (FWS 2008). O'Shea & Clark (2002) suggested that insecticides may be an important contributor to bat declines. Eidels at al. (2007) analyzed bats and guano from Indiana and found insecticide residues in all of the samples. Nevertheless, the role of insecticides in the decline of bats remains unclear, as no recent toxicological studies on bats have been published.

Most bat species in North America feed on large quantities of insects (Whitaker 1996). For example, a single little brown bat (*Myotis lucifugus*) consumes as much as 28–85% its body weight in insects per day (Edythe et al. 1977; Kurta et al. 1989). Feeding on large quantities of insects may bring bats into contact with a wide range of insecticides. Testing of bats for insecticide residues could promote

Correspondence: Ronny R. Eidels, HMRI Bioresources, University of Newcastle, University Drive, Callaghan NSW 2308, Australia, Phone: +61–2–40420062, Fax: +61–2–40420021, (e-mail: Ronny.Eidels@newcastle.edu.au).

detection of insecticides in the environment, including those that have not been documented to bioaccumulate in bats.

The present study was designed to identify which insecticides can be found in free ranging bats in Indiana. Identifying insecticides in free ranging bats is challenging because bats are likely to be exposed to insecticides while foraging away from their roosts which may cause them to be incapacitated and not able to return to their roosts. Therefore, sampling bats using the standard methods, such as netting major fly routes or collecting bats from their roosts, is not likely to provide much information on the more acute and toxic insecticides. To increase the prevalence of affected bats in the sample population, bats that were already distressed (i.e. sick, dead or otherwise incapacitated) were tested. Throughout the years bats from Indiana were collected by the public and submitted to the *Indiana State Department of Health* rabies laboratory for rabies testing because they were distressed. Only about 5% of these bats were found to be rabid (Whitaker & Douglas 2006) leaving the cause for the incapacitation of about 95% of them unexplained. Eidels et al. (2007) examined nine of these bats and found that all nine contained insecticide residues. In the current study, non-rabid bats from the rabies laboratory were tested. Whole body analyses were used to identify insecticide residues in the bats. To complete the residue analyses cholinesterase (ChE) bioassays were performed. These bioassays measured the effect of ChE inhibiting insecticides (organophosphorus compounds and carbamates) on the bats' brains. ChE inhibitors are labile in living tissues and therefore may not be detected in residue analysis (Hill 1989, 1995; O'Shea & Clark 2002). The bioassays were conducted to identify exposed bats in which residues were not detected.

METHODS

Animals.—A total of 332 bats were collected from throughout Indiana by the public between September 2005 and December 2007. Bats were submitted to the *Indiana State Department of Health* rabies laboratory for rabies testing and found non-rabid. Brains were removed and a portion of each brain was used for rabies testing. The remaining brain was kept for cholinesterase (ChE) bioassays. Non-rabid bats (stored in Ziploc® bags) and brains from non-rabid bats (stored in 1.5 ml Eppendorf tubes) were transported frozen to *Indiana State University* where they were kept frozen at −60°C until analysis. 40 of these bats were collected from Lake, Porter and LaPorte Counties in North West Indiana (Figure 1). Lake, Porter and LaPorte Counties cover an area of approximately 3,600 km^2 where the land is in urban, industrial and agricultural use. The species and sex composition of the 40 bats is outlined in Table 1.

Figure 1.—Indiana map with Lake, Porter and LaPorte Counties, Northern Indiana colored grey. Map by the Indiana Business Research Center, January 2004 http://www.stats.indiana.edu/.

Whole body residue analysis.—Whole body chemical analysis of the 40 bats collected from Lake, Porter and LaPorte Counties (Table 1) was conducted to identify 35 insecticides from four groups (Table 2).

Shortly before analysis, bats were weighed and identified (species, sex and age: juvenile or adult, determined by forearm length). Skin and wings were removed and the carcasses sent on dry ice in 60 ml short wide-mouth clear glass jars (I-CHEM Brand) to *Southern Illinois University Carbondale* where they were analyzed for insecticide residues.

Analytical methods: Samples were processed in two batches of 20 bats. The first 10 samples

Table 1.—Species and sex composition of the 40 bats collected from Lake, Porter and LaPorte Counties. One of the bats, whose sex was not determined, was a new born.

Species	Males	Females	Sex not determined	Total
Big brown bats (*Eptesicus fuscus*)	20	13	2	35
Silver-haired bats (*Lasionycteris noctivagans*)	2	1		3
Red bats (*Lasiurus borealis*)	1	1		2
Total	23	15	2	40

in batch 1 were extracted using approximately 5 g of tissue per specimen. Because of the high lipid content of the bats, the remaining 10 samples in batch 1 and the second batch of 20 bats were extracted using approximately 2 g of tissue.

Gas chromatography with electron capture detector (GC-ECD) and nitrogen-phosphate detector (GC-NPD) (Agilent Technologies, Palo Alto, CA, USA) were used for insecticide analysis (Belisel & Swineford 1988; You & Lydy 2004). Analyses of eight pyrethroid, 19 organochlorine, six organophosphate and two carbamate insecticides (Table 2) were performed. After homogenization, bat samples were extracted with a mixture of methylene chloride : acetone (1:1, volume: volume) using an accelerated solvent extractor (ASE 200, Dionex, Sunnyvale, CA, USA). Extracts were cleaned and fractionated by tandem solid phase extraction into different groups for instrumental analysis (You & Lydy 2004). A sub-sample of bat tissue was used to measure lipid content spectrophotometrically with vanillin-H_3PO_4 reagent after acid-digestion (Van Handel 1985). Lipid analyses were performed using three replicates.

The carbamate insecticide carbaryl (1-naphthyl methylcarbamate) could not be quantified in the bat samples due to a matrix enhancement in the response factor seen on the gas chromatograph likely due to the high lipid concentrations in the bats. To offset this situation, standards were made in 0.05% and 0.1% corn oil in an attempt to mimic the matrix enhancement that occurred in the bats.

Quality control measurements including accuracy (percent recoveries) and precision (relative standard deviations) were calculated for each batch of 20 bat samples in addition to the processing of method detection limits (sensitivity), a blank, lab control spike, matrix spike and matrix spike duplicate.

Brain cholinesterase activity and reactivation testing.—Cholinesterase (ChE) activity was measured in the brains of 332 bats from throughout Indiana. Frozen brains were homogenized in the presence of 7.4 pH Tris buffer using laboratory homogenizer (Polytron PT 10/35 with Power Control Unit PCU-11, Kinematica, Inc., Bohemia, New-York, USA). For each sample, brain ChE activity was measured (Fairbrother et al. 1991; Ellman et al. 1961), thereafter, ChE reactivation tests were performed. Cholinesterase activity was measured colorimetrically using a modification of the Ellman assay (Fairbrother et al. 1991; Ellman et al. 1961). Cholinesterase activity was expressed as micromoles of acetylthiocholine iodide (substrate) hydrolyzed per minute (i.e. units) per gram of brain tissue wet weight (units/g).

The ChE reactivation tests measure increase of brain ChE activity in exposed bats after

Table 2.—List of insecticides tested in residue analysis.

Group	Compound
Pyrethroids	bifenthrin, lambda-cyhalothrin, cyfluthrin, cypermethrin, permethrin, esfenvalerate, deltamethrin and fenpropathrin
Organochlorines	alpha-BHC, beta-BHC, gamma-BHC, delta-BHC, p,p'-DDE, p,p'-DDD, p,p'-DDT, aldrin, gamma-chlordane, alpha-chlordane, dieldrin, endrin, endrin ketone, endosulfan I, endosulfan II, endosulfan sulfate, heptachlor, heptachlor epoxide and methoxychlor
Organophosphates	chlorpyrifos, malathion, tebupirimphos, dichlorvos, diazinon, terbufos
Carbamates	carbaryl, carbofuran

removing the insecticide molecules that bonded and neutralized the brain ChE enzymes. Each homogenized brain sample was divided into five aliquots. Presence of cholinesterase inhibition due to organophosphate (OP) exposure was identified using a method described by Martin et al. (1981). Two aliquots from each brain sample were incubated for 30 minutes at 24°C: one (the test aliquot) in the presence of pyridine 2-aldoxime methiodide (2-PAM) and the other (the control aliquot) in a 7.4 pH Tris buffer. Thereafter, ChE activity was measured simultaneously in both aliquots. If OP inhibited enzymes were present in the test aliquot, 2-PAM should remove the bound OP and, by that, increase the brain ChE activity (Fairbrother et al. 1991). To identify inhibition of ChE caused by exposure to a carbamate insecticide, a method described by Hunt & Hooper (1993) was used. Two aliquots from each homogenized brain sample were further diluted with 7.4 pH Tris buffer solution and incubated for 3 hours, one (the test aliquot) at 37°C and the other (the control aliquot) at 4°C. If carbamates were present in the test aliquot, incubation at 37°C should accelerate the diffusion of insecticide molecules to the buffer solution and, therefore, accelerate the rate of spontaneous reactivation of ChE in that aliquot (Hunt & Hooper 1993). For both OP and carbamate reactivation tests, brain ChE reactivation was considered significant if the activity of the test aliquot was at least 5% higher than the activity of the control aliquot; and a one tailed Student's t-test showed a statistically significant difference ($P<0.05$) between the activity level measured in the two aliquots (Hunt & Hooper 1993).

The ChE analyses were performed using three replicates and the mean value of the replicates was used in all calculations. An aliquot with a coefficient of variation greater than 5% among its replicates was rerun. For each batch of samples (30 aliquots), a blank and a known standard were processed. Reagents were made fresh daily.

RESULTS

Bats tested in this study had been submitted to the rabies laboratory throughout the year, with 35% of them submitted during the peak activity period in August. Of the tested bats, 88% were big brown bats (*Eptesicus fuscus*). Big brown bats live mostly around human habitat, therefore they have high potential to be encountered by members of the public as well as exposed to pesticides. Detailed results including frequencies and concentration ranges of insecticide residues in the tested bats are presented in Table 3.

Whole body residue analysis.—The average lipid concentration in the 40 bats tested was 7.07% (range: 1.52–25.5%). The median lipid concentration was 5.76%.

Organochlorine insecticides (OCs) were found in 97.5% of the 40 bats tested (Table 3). They were found in both sexes, across species and throughout the year. The following OCs were detected: Dieldrin ((1a*R*,2*R*,2a*S*,3*S*,6*R*,6a*R*,7*S*,7a*S*)-3,4,5,6,9,9-hexachloro-1a,2,2a,3,6,6a,7,7a-octahydro-2,7:3,6-dimethanonaphtho[2,3-*b*]oxirene), DDT (dichlorodiphenyltrichloroethane 1,1,1-trichloro-2,2-di(4-chlorophenyl)ethane), DDE (Dichlorodiphenyl-dichloroethylene (2,2-*bis*-(4-chlorophenyl)-1,1-dichloroethene)), DDD (Dichlorodiphenyl-dichloroethane 1-chloro-4-[2,2-dichloro-1-(4-chlorophenyl)ethyl]benzene), heptachlor epoxide (Epoxyheptachlor 1,4,5,6,7,8,8-Heptachloro-2,3-epoxy-3a,4,7,7a-tetrahydro-4,7-methanoindan), endosulfan (6,7,8,9,10,10-Hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepine-3-oxide) and alpha-chlordane (α-Octachloro-4,7-methanohydroindane).

Five (12.5%) of the bat samples contained pyrethroid residues and all contained detectable concentrations of OCs. Two of the five also contained organophosphates. All five bats were male *E. fuscus* submitted to the rabies lab between June and September (2005–2007 combined). Two bats were found in Porter County, two in Lake County and one in LaPorte County. Their lipid concentration ranged between 2.42–14.1% (mean = 7.6%, median = 7.72%).

Ten of the bats (25%) had detectible levels of OPs. Of these, seven were found between May and August, one was found in March and two in November–December. Five were females and five males. Five were found in Lake County and five in Porter County. Lipid concentrations of these ten bats ranged between 2.67–10.3% (mean = 9.1%, median = 6.53%). Nine of the ten bats contained diazinon (*O*,*O*-Diethyl *O*-[4-methyl-6-(propan-2-yl)pyrimidin-2-yl] phosphorothioate). Of these nine, eight were *E. fuscus* and one was a red bat (*Lasiurus borealis*). Two of the bats that contained diazinon also contained pyrethroids. The one bat that contained chlorpyrifos (*O*,*O*-Diethyl *O*-(3,5,6-trichloro-2-pyridinyl) phosphorothioate

Table 3.—Frequency and concentration of insecticides in bats (n = 40) from Lake, Porter and LaPorte Counties, Indiana. dnq – detected, not quantified.

Insecticides	Frequency of contaminated samples	% Contaminated samples	Pesticide body residues mg/kg wet weight			Reporting limit mg/kg wet weight
			Range	Average	Median	
Organochlorines	**39**	**97.5**				
dieldrin	36	90	0.01–1.5	0.15	0.09	0.005
DDE	36	90	0.06–5.16	0.94	0.37	0.005
DDD	14	35	0.01–0.31	0.09	0.03	0.005
DDT	15	37.5	0.02–0.53	0.13	0.07	0.005
heptachlor epoxide	9	22.5	0.01–0.78	0.20	0.06	0.005
endosulfan I	3	7.5	0.01–0.02	0.02	0.02	0.005
alpha-chlordane	1	2.5	0.04	0.04	0.04	0.005
Pyrethroid	**5**	**12.5**				
bifenthrin	1	2.5	0.37	0.37	0.37	0.005
lambda isomer	1	2.5	0.01	0.01	0.01	0.005
lambda-cyhalothrin	1	2.5	0.18	0.18	0.18	0.005
permethrin	1	2.5	0.02	0.02	0.02	0.005
cypermethrin	1	2.5	0.00002	0.00	0.00	?
cypermethrin 1	1	2.5	0.01	0.01	0.01	0.005
cypermethrin 3	1	2.5	0.005	0.00	0.00	0.005
cypermethrin 2 (+4)	1	2.5	0.004	0.00	0.00	?
esfenvalerate	2	5	0.01–0.03	0.02	0.02	0.005
esfenvalerate 1	1	2.5	0.01	0.01	0.01	0.005
esfenvalerate 2	1	2.5	0.03	0.03	0.03	0.005
Organophosphates	**12**	**30**				
chlorpyrifos	1	2.5	0.12	0.12	0.12	0.005
diazinon	9	22.5	0.03–0.81	0.43	0.60	0.025
Carbamates	**1**	**2.5**				
carbaryl	1	2.5	dnq			0.025

was *E. fuscus*. All 10 bats also contained OCs. None of the 10 bats showed significant reactivation of ChE activity after incubation with 2-PAM.

Carbaryl (1-naphthyl methylcarbamate) was detected in one bat, though it could not be quantified. In an attempt to measure the quantity of the carbaryl residues, standards were made in corn oil. Adding lipid to the standards did increase the response factor, however, it was not sufficient to account for the enhancement seen in the bat specimen. The exposed individual was a female *L. borealis* found in July in Porter County. Its lipid concentration was 3.12%. In addition to carbaryl, this bat had diazinon and OC residues. In the brain ChE reactivation tests, this bat showed significant spontaneous reactivation of at least 5% of its brain ChE function.

Statistical analysis: Since OC insecticides were found in all of the sampled bats but one, there was no need to conduct χ^2 statistical tests to identify relationships between exposure to OCs and other factors of this sample such as geographic origin of the bats, their sex or species. This sample indicates that all the geographic areas tested had equal OC contamination levels. Similarly both sexes and all species in the sample were equally contaminated. Therefore this insecticide group was not included in the following statistical analysis. There was no significant relationship between insecticide exposure and the county of the bats' origin (χ^2 (2)=0.57, p=0.75); between insecticides and sex (Chi square(1)=0.34, p=0.56) and between insecticides and season (χ^2(3)= 2.39, p=0.5). This implies that insecticide residues were found equally in all three counties; that male and female bats were equally exposed to contamination; and that bat exposure to insecticides was equally distributed throughout the year. The results of these analyses should be interpreted with care because of the small sample size (n=40) of this

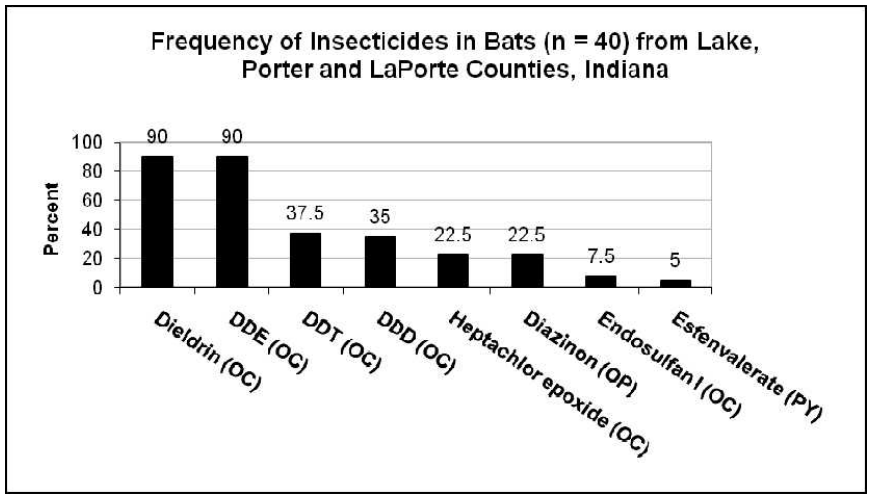

Figure 2.—Frequencies of the main insecticides detected in the 40 bats tested. Bats were collected from Lake, Porter and LaPorte Counties, Indiana by the public and submitted to the *Indiana State Department of Health* rabies laboratory between September 2005 and December 2007. The letters in parentheses represent the insecticide group: OC – organochlorines, OP – organophosphates, PY – pyrethroids.

part of the survey (Table 1). Pyrethroid and cholinesterase insecticide residues were not detected in any of the individuals of *Lasionycteris noctivagans* tested. The ChE inhibitors (diazinon and carbaryl) were found in one of the two *L. borealis* tested.

Bats with insecticide mixtures and heavier residue burdens: 37 of the 40 bats tested (92.5%) had residues of more then one insecticide; of these, 70.3% (26 bats) had residues of three or more insecticides. Thirteen bats (32.5%) had residues of pyrethroids or/and ChE inhibitors; all of them had residues of OC as well. Sixteen (40%) of the bats had residues of three or more insecticides with relatively high residual load of at least one insecticide. The average lipid concentration in these bats was 8.51% (range of 1.77–23.2%) and the median was 6.92%. There was no significant relationship between the distribution of the more heavily contaminated bats and county (χ^2 (2) = 2.4, p = 0.3); sex (χ^2 (1) = 0.002, p = 0.97) or season (χ^2 (3) = 2.69, p = 0.44). The results of these analyses should be interpreted with care because of the small sample size.

Brain ChE activity and reactivation testing.—Of the 332 bat brains tested six (1.8%) showed significant recovery of brain ChE function after incubation with 2-PAM. As part of this survey, 33 of the 332 bats whose brains were tested for ChE activity and reactivation were also analyzed for pesticide body residues. While 10 of these 33 bats contained residues of the OPs diazinon and chlorpyrifos, none of them demonstrated significant ChE reactivation after incubation with 2-PAM. One hundred and fifty five (46.67%) of the samples showed significant spontaneous recovery of 5% or more. No significant relationships were found between spontaneous reactivation and sex (χ^2 (2) = 0.63, p = 0.73), county (for counties with sample size of 7 or more bats, χ^2 (10) = 9.56, p = 0.48) or season (χ^2 (3) = 1.75, p = 0.63).

DISCUSSION

Overall, the majority of the bat samples had detectable concentrations of dieldrin and DDE, while many samples also had detectable concentrations of DDT, DDD, heptachlor epoxide and diazinon (Figure 2). Organochlorines were by far the major insecticides detected and were found in 97.5% of the bat samples. Chronic exposure to insecticides from this group may cause a variety of clinical effects based on the insecticide and the species examined. In mammals, signs of OC exposure include anorexia, muscular weakness, hyper-excitability, muscle twitching, ataxia, visual blurring, loss of consciousness and death (Ecobichon 2001). Chronic exposure to OCs may hamper the ability of bats to enter torpor and to forage effectively and therefore to control their ener-

getic requirement. Energetic deficiency may lead to inability of female bats to support their young. In extreme cases it may cause indirect death of affected bats. Chronic exposure to OCs may also lead to reproductive disorders (Ecobichon 2001) and may cause further reduction of the already low reproductive rate of bats (one to two young per year by most species, three to four in red bats).

Due to the high persistence of OCs and their tendency to bioaccumulate, the U.S. Environmental Protection Agency (EPA) had imposed restrictions on the use of most OCs since the 1970s and early 1980s. By 1988, only four OCs were still used in agriculture in the USA and included dicofol (2,2,2-trichloro-1,1-bis(4-chlorophenyl)ethanol), endosulfan, lindane ((1*r*,2*R*, 3*S*,4*r*,5*R*,6*S*)-1,2,3,4,5,6-hexachlorocyclohexane) and methoxychlor (1,1,1-Trichloro-2,2-bis(4-me thoxyphenyl)ethane). The use of dieldrin, DDT, DDE and DDD was restricted in the 1970s (Ecobichon 2001; Harte 1991; Nowell et al. 1999; USGS 1999). Therefore, bat residues from these insecticides are the result of continued bioaccumulation and persistence of OC pesticides that were applied more than 30 years ago. Because almost all bats in the sampled population contained OCs, it is likely that many of the bats populating the sampled areas, including several species and both sexes are contaminated with these chemicals.

Forty percent of the 40 bats tested had residues of three or more types of insecticides. All these bats contained OCs and 81% of them contained more than one type of organochlorines. Considering the high persistence of OCs it is not surprising that many of these compounds are still common in the environment and become available to bats.

Another prevalent insecticide was diazinon. It was detected in 22.5% of the bats. Diazinon is an organophosphate pesticide used in agricultural applications to control a variety of insects. It is registered to control foliage and soil insects as well as pests of many fruit, nut, vegetable, and field crops. Diazinon is also used in cattle ear tags. Up until the early 2000s, diazinon was one of the most widely used insecticides in the U.S. for household, lawn and garden pest control (up to 3,500 metric tons used each year) as well as agricultural pest control (about 30% of all use). By the end of 2004, EPA phased out and eliminated all residential uses of this insecticide, but approval for agricultural uses continued. The EPA risk assessment conducted in 2002, stated that diazinon posed unacceptable risks to birds and other wildlife species. As a result, the agency announced in 2006 its plan to phase out within 2–5 years certain agricultural crop uses, phase out the use of a granular formulation and aerial applications, and reduce the overall amount and frequency of use (EPA 2006, 2008).Approximately 6,000 metric tons of the active ingredient diazinon were still used annually on agricultural sites in 2008 (EPA 2008).

It may be that finding diazinon residues in such high rates while its use is being significantly reduced, implies that this chemical is more persistent in the environment than thought. Ferrando et al. (1992) tested persistence of various insecticides (organochlorines, OPs and carbamates) in unfiltered lake water (pH = 9.0 ± 0.5) stored at 22°C. The most persistent insecticides in their study were diazinon (half life of 71 h in natural water) and thiobencarb (*S*-[(4-chlorophenyl)methyl] *N,N*-diethylcarbamothioate), which is a carbamate with half life of 74 h in natural water. This implies that ChE inhibitors can persist in surface water for days after application and longer during the colder months (fall and spring). Contaminated water after ChE inhibitor application may therefore become another exposure medium of diazinon to wildlife.

The other ChE inhibitors identified in the sampled bats were chlorpyrifos and carbaryl. Finding ChE inhibitors in bat samples is surprising because they are short-lived and do not persist in the living body. Several authors have suggested that since ChE inhibitors do not tend to bioaccumulate in living tissue, their presence is indicative of recent exposure prior to death (Hill 1989, 1995; O'Shea & Clark 2002). Nevertheless, the presence of ChE inhibitor residues in the absence of demonstrated ChE inhibition supports, but does not confirm causation in a specific mortality (Eidels et al. 2007).

Pyrethroid insecticides entered the marketplace in 1980. Within two years they comprised more than 30% of the worldwide insecticide usage (Ecobichon 2001). These are synthetic chemicals whose structures mimic the natural insecticide pyrethrins. Pyrethrins are produced by the flowers of the Pyrethrum plants (*Chrysanthemum cinerariaefolium* and *C. coccineum*).

They are highly biodegradable and break down easily when exposed to sunlight. Pyrethroids were manufactured to persist longer in the environment than pyrethrins. Pyrethroids bind sodium channels on the axon membrane of nerve cells. As a result, the channels are unable to close normally which leads to continuous nerve stimulation. Clinical signs of pyrethroid exposure include tremors and inability to produce coordinated movement (Valles & Koehler 1997). The EPA (2006) decision to phase out some of the OPs and restrict the use of others led to their gradual replacement with pyrethroids. Pyrethroids were detected in five of the 40 bats tested; all were male *E. fuscus*. Three of these bats contained more than one type of pyrethroid. None of the individual insecticides in this group occurred in more than two bats. Two of the five bats with pyrethroid residues contained diazinon residues as well and all five had OC residues.

Ten (77%) of the 13 bats containing ChE inhibitors and/or pyrethroid residues were collected between May and September which are the active months for the bats and also the seasons in which insecticides are more likely to be applied. Detection of pesticides that do not persist in living tissues is expected to be highest immediately after application.

Only 1.8% of the 332 bats tested for brain ChE reactivation showed significant recovery of brain ChE activity. Furthermore, of the 10 bats that their whole body residue analysis confirmed that they contained OP residues, none showed significant recovery of brain ChE activity after incubation with 2-PAM. This raises the question whether the reactivation tests identified all the bats that were exposed to OP. If the initial dose of OP is too small to affect the brain or the inhibited enzymes become resistant to reactivation, reactivation tests will not identify exposure to OPs (Wilson et al. 1992). OP inhibited ChE may become resistant to reactivation by undergoing 'aging' process. Aging occurs when conformational change in the molecular structure of the organophosphate occurs after the initial organophosphate cholinesterase bonds are formed. This structural change increases the strength of the organophosphate-cholinesterase bond and makes the complex irreversibly bonded (Johnson et al. 2000). Aging is associated with dealkylation (the removal of alkyl group, an organic molecule made up of chains of carbon and hydrogen atoms joined by single bonds) by C-O or P-O fission (Beauregard et al. 1981). This process can occur within hours to days after the initial effect, depending upon the structure of the OP, the pH and the temperature (Johnson et al. 2000).

If aging occurred, incubation with 2-PAM of brains from even severely poisoned animals with significant inhibition of brain ChE will not yield enzyme reactivation. Wilson et al. (1992) measured brain ChE reactivation after incubation with 2-PAM in quail poisoned with parathion. Reactivation persisted for two days at ambient temperature. After four days at ambient temperature, there was no reactivation of brain enzyme in the birds. Bats that were submitted to the rabies laboratory were already dead. Storage conditions prior to submission were unknown. It could be that if the bats were not frozen immediately after death, aging of their brain enzymes occurred. In that case, enzyme reactivation may not be a suitable method to identify OP exposure. Due to potential false negatives no specific conclusions could be made with regard to exposure of bats to OPs in Indiana.

Cholinesterase enzymes inhibited by carbamates regain their full activity as the enzyme-carbamate bond degrades. Hunt & Hooper (1993) developed an assay that identifies this spontaneous reactivation of brain ChE as an indicator of carbamate poisoning. The problem rose when the same authors identified similar spontaneous reactivation of brain ChE activity in brains that were not exposed to carbamates (Hunt & Hooper 1993). They attributed this reactivation to a slow release of divalent cations (Ca^{2+} or Mg^{2+}) from cellular membranes fractured during the brain homogenization process. To capture these cations they added the chelating agent EDTA (ethylenediaminetetraacetic acid, tetrasodium salt) to the brain homogenate. This effectively eliminated the increase in ChE activity in non-contaminated brains of birds and mammals tested by these authors. Hunt & Hooper (1993) also incorporated EDTA in their spontaneous reactivation assay (Hunt & Hooper 1993).

For this work, the Hunt & Hooper's (1993) reactivation assay was used to identify carbamate poisoning and EDTA was added in the concentration recommended by these authors (and later in higher concentrations) to all brain samples. Of the 332 samples tested, 46.67%

showed significant recovery of at least 5% of brain ChE activity. This reactivation occurred equally throughout the year, in both males and females and across the different counties in Indiana (with a sample size of 10 bats or more). Such high exposure rates to pesticides that do not persist in the environment (Hill 1989, 1995; O'Shea & Clark 2002) can only occur shortly after a vast application of large quantities of these insecticides. Because of the unrealistically large percentage of bats that demonstrated significant spontaneous reactivation and the fact that some of these bats were found during winter when insecticides are rarely in use we suspect false positive errors in our testing. We shall describe shortly the measures that may be taken against such bias. Spontaneous increases in ChE activity in bat brains may result from a process other than the one suggested by Hunt & Hooper (1993). If that is the case, the Hunt & Hooper (1993) reactivation assay should not be used without adjustments to identify carbamate poisoning in bats. Nevertheless, while the option of a false positive error exists, no conclusions could be made based on these results regarding the exposure of bats to carbamates in Indiana.

The results of this research suggest that even today, approximately 30 years after OC insecticides were phased out, bats in the Indiana Great Lakes region are still exposed to these insecticides. To fully appreciate the implications, the effects of chronic exposure of bats to OCs should be further explored. The presence of ChE inhibiting insecticides in bats from the Great Lakes region was also demonstrated. Clark (1986) orally dosed bats and mice with the OP methyl parathion and found that although the LD_{50} for bats was 8.5 times the LD_{50} for mice; bats lost coordination after one hour and were still unable to right themselves at the end of the study, 24 hours after dosing. Mice, on the other hand, recovered back to normal within 2–3 hours after the OP administration. Clark (1986) suggested that free ranging bats that are exposed to OPs, may lose coordination, fall to the ground and die. To assess the risk that ChE inhibitors pose to bats, controlled dosing studies as well as field studies testing free ranging bats following insecticide applications are required. This information, combined with statistical data on the use of ChE inhibitors should allow an estimation of the overall impact of these pesticides on bats. Pyrethroids and carbamates were also detected in bats as part of the residue analysis performed in the current study. These results are among the first to reported residue detections of pesticides from these groups in bats.

This work is the first step in investigating the impact of insecticides on bats. Its objective was to identify the major insecticides to which bats are exposed in order to focus further investigation on these specific pesticides. Because the sampling of bats was not random, no assumptions could be made with regards to the extent of exposure in the overall bat population. Nevertheless, testing already distressed bats allowed identification of insecticide residues in these animals without sacrificing healthy free ranging bats.

ACKNOWLEDGMENTS

This study was supported by the U. S. Environmental Protection Agency, Great Lakes National Program Office, Bat Conservation International and the U.S. Fish and Wildlife Service. The authors would like to thank Louis Douglas, Ami Eidels, Charles Sprague, Vergel Santos, Diana Hews and Diane Henshel for their assistance.

LITERATURE CITED

Belisle, A.A. & D.M. Swineford. 1988. Simple, specific analysis of organophosphorous and carbamate pesticides in sediments using column extraction and gas chromatography. Environmental Toxicology and Chemistry 7:749–752.

Beauregard, G., J. Lum & B.D. Roufogalis. 1981. Effect of histidine modification on the aging of organophosphate-inhibited acetylcholinesterase. Biochemical Pharmacology 30:2915–2920.

Clark, D.R., Jr. 1986. Toxicity of methyl parathion to bats: mortality and coordination loss. Environmental Toxicology and Chemistry 5:191–195.

Ecobichon, D.J. 2001. Toxic effects of pesticides. Pp. 769–787. *In* Casarett & Doull's toxicology (C.D. Klaassen, ed.). McGraw-Hill Medical Publishing Division, Chicago, Illinois.

Edythe, L.P. & H.K. Thomas. 1977. Feeding strategies of the little brown bat, Myotis lucifugus, in southern New Hampshire. Ecology 58:775–786.

Eidels, R.R., J.O., Jr. Whitaker & D.W. Sparks. 2007. Insecticides Residues in Bat and Guano from Indiana. Proceedings of the Indiana Academy of Science 116(1):50–57.

Ellman, G.L., D.K. Courtney, V., Jr. Andres & R.M. Featherstone. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochemical Pharmacology 7:88–95.

Fairbrother, A., B.T. Marden, J.K. Bennett & M.J. Hooper. 1991. Methods used in determination of cholinesterase activity. Pp. 35–71. *In* Cholinesterase Inhibiting Insecticides: Their Impact on Wildlife and the Environment (P. Mineau, ed.). Elsevier, New York.

Ferrando, M.D., V. Alarcón, A. Fenández-Casalderrey, M. Gamón & E. Andreu-Moliner. 1992. Persistance of some pesticides in the aquatic environment. Bulletin of Environmental Contamination and Toxicology 48(5):747–755.

Harte, J. 1991. Toxics A to Z: a guide to everyday pollution hazards. University of California Press, California.

Hill, E.F. 1989. Divergent effects of postmortem ambient temperature on organophosphorus and carbamate inhibited brain cholinesterase activity in birds. Pesticide Biochemistry and Physiology 33:264–275.

Hill, E.F. 1995. Organophosphorus and pesticides. Pp. 243–274. *In* Handbook of Ecotoxicology (D.J. Hoffman, B.A. Rattner, G.A., Jr. Burton & J., Jr. Cairns, eds.). Lewis Publishers, Boca Raton, Florida.

Hunt, K.A. & M.J. Hooper. 1993. Development and optimization of reactivation techniques for carbamates-inhibited brain and plasma cholinesterases in birds and mammals. Analytical Biochemistry 212:335–343.

Johnson, M.K., D. Jacobsen, T.J. Meredith, P. Eyer, A.J. Heath, D.A. Ligtenstein, T.C. Marrs, L. Szinicz, J.A. Vale & J.A. Haines. 2000. Evaluation of antidote for poisoning by organophosphorus pesticides. Emergency Medicine 12:22–37.

Kunz, T.H. & M.B. Fenton. 2003. Bat Ecology. University of Chicago Press, Chicago, Illinois.

Kurta, A., G.P. Bell, K.A. Nugy & T.H. Kunz. 1989. Energetics of pregnancy and lactation in free ranging little brown bats (Myotis lucifugus). Physiological Zoology 62(3):804–818.

Lee, Y.F. & G.F. McCracken. 2005. Dietary Variation of Brazilian Free-Tailed Bats Links to Migratory Populations of Pest Insects. Journal of Mammalogy 86(1):67–76.

Martin, A.D., G. Norman, P.I. Stanley & G.E. Westlake. 1981. Use of reactivation techniques for the differential diagnosis of organophosphorus and carbamate pesticide poisoning in birds. Bulletin of Environmental Contamination and Toxicology 26:775–780.

Nowell, L.H., P.D. Capel & P.D. Dileanis. 1999. Pesticides in stream sediment and aquatic biota distribution, trends and governing factors. U.S. Geological Survey, Sacramento, California.

O'Shea, T.J. & D.R., Jr. Clark. 2002. An overview of contaminants and bats, with special reference to insecticides and the Indiana bat. Pp. 237–253. *In* The Indiana bat: biology and management of an endangered species (A. Kurta & J. Kennedy, eds.). Bat Conservation International, Austin, Texas.

U. S. Environmental Protection Agency. 2006. Reregistration eligibility decision (RED) for diazinon. http://www.epa.gov/pesticides/reregistration/REDs/diazinon_red.pdf.

U.S. Environmental Protection Agency. 2008. Pesticides: Registration Diazinon IRED facts. http://www.epa.gov/pesticides/reregistration/REDs/factsheets/diazinon_ired_fs.htm.

U.S. Fish & Wildlife Service. 2008. Indiana Bat Revised* 2007 Rangewide Population Estimate. http://www.fws.gov/midwest/endangered/mammals/inba/inba_2007pop.html.

U.S. Geological Survey. 1999. The quality of our nation's waters – U.S. Geological Circular 1225 - nutrients and pesticides. U.S. Geological Survey. Reston, Virginia. http://pubs.usgs.gov/circ/circ1225/index.html.

Valles, S.M. & P.G. Koehler. 1997. Insecticides used in the urban environment: mode of action. ENY282, Entomology and Nematology Department. Florida Cooperative Extension Service, Institute of Food and Agricultural Sciences, University of Florida. Gainesville, Florida.

Van Handel, E. 1985. Rapid determination of total lipids in mosquitoes. Journal of American Mosquito Control Association 1:302–304.

Whitaker, J.O., Jr. 1995. Food of the big brown bat, Eptesicus fuscus, from maternity colonies in Indiana and Illinois. American Midland Naturalist 134:346–360.

Whitaker, J.O., Jr. 1996. National Audubon Society® field guide to North American mammals. Alfred A. Knopf Inc, New York.

Whitaker, J.O., Jr., V., Jr. Brack & J.B. Cope. 2002. Are bats in Indiana declining? Proceedings of the Indiana Academy of Science 1:95–106.

Whitaker, J.O., Jr. & L.R. Douglas. 2006. Bat Rabies in Indiana. Journal of Wildlife Management 70(6): 1569–1573.

Whitaker, J.O., Jr. & W.J., Jr. Hamilton. 1998. Mammals of the Eastern United States. Cornell University Press, Ithaca, New York.

Wilson, B.W., M.J. Hooper, M.E. Hansen & P.S. Nieberg. 1992. Reactivation of organophosphorous inhibited AChE with oximes. Pp. 107–137. *In* Organophosphates Chemistry, Fate and Effects (J.E. Chambers & P.E. Levi, eds.). Academic Press Inc, San Diego, California.

You, J. & M.J. Lydy. 2004. Simultaneous determination of pyrethroid, organophosphate and organochlorine pesticides in fish tissue using tandem solid-phase extraction clean-up. International Journal of Environmental Analytical Chemistry 84:559–571.

Manuscript received 19 August 2012, revised 10 January 2013.