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Organophosphate Insecticide Residues in Bats From Indiana

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Bats are metabolically some of the most complex vertebrates and have seasonally very high energy demands due to among other things their small size and mode of locomotion (flight). This requires a greater food intake and increases the potential for exposure to dietary toxicants (Clark and Shore 2001). During lactation, little brown myotis (*Myotis lucifugus*) can consume up to 85 % of their body weight each night (Kurta et al. 1989). Food consumption rates are also high in the spring prior to migration and prior to hibernation when bats need to build up energy reserves to survive hibernation. In addition, bats are long lived. Davis and Hitchcock (1995) reported the recapture of a male little brown myotis that was banded in 1961, 34 years earlier. Indiana myotis have been known to live up to 20 years (Whitaker and Hamilton 1998). Long life span allows more time for contact and accumulation of insecticides and other toxicants.

Apart from being an important part of the ecosystem, insectivorous bats are the main predators on nocturnal insects and they consume numerous agricultural pests (Whitaker and Hamilton 1998). Lee and McCracken (2005) examined food habits of Brazilian free-tailed bats (*Tadarida brasiliensis*) in central Texas. They found that crop pests comprise much of the bats diet and suggested that bats provide natural pest control. However many North American bats are declining, including the federally endangered Indiana myotis (*Myotis sodalis*). It's total population was estimated to be approximately 380,000 bats in 2001, was is down from an estimated 880,000 bats in 1960 (Clawson 2002). Whitaker et al. (2002) indicated that the evening bat (*Nycticeius humeralis*), the little brown myotis, the eastern red bat (*Lasiurus borealis*) and the hoary bat (*Lasiurus cinereus*) are declining in Indiana. Habitat decrease and vandalism in hibernacula are parts of the reasons for this decline. Another reason may be environmental contaminants such as pesticides. It is clear that several organochlorine pesticides including dieldrin, heptachlor, DDE, DDT have been implicated in localized bat mortality incidents (Clark et al. 1978, 1980, Clark 1981). However, it is unknown how widespread these types of incidents may have been in the past or what relation these few documented incidents have had to range-wide bat populations. It is even less clear what effects present day insecticides might have on bats. The decline of insectivorous bats might be related to decrease in food availability, sublethal toxic effects, or perhaps both. Regardless, reductions in insectivorous bats is certainly not a benefit to agricultural pest managers.

Cholinesterase inhibiting insecticides, organophosphates (OP) and carbamates (CA), are currently the most widely used insecticides (Grue et al. 1997). They are highly toxic but not highly fat soluble, and are not previously reported to accumulate in living tissues (O'Shea and Clark 2002; Hill 1995) or in food chains (Schmidt et al. 2002). Hoffman et al. (2001) describes OPs and CAs as inhibitors of the enzyme cholinesterase (ChE). ChE hydrolyzes the neurotransmitter acetylcholine in nervous tissue. Clinical signs of toxicity include salivation, lacrimation, urination, defecation, tremor, convulsions and eventually paralysis (Grue et al. 1997). Death occurs due to respiratory failure and asphyxia (Wilkinson, 1976). In addition to neurotoxic effects, anti ChE compounds have been shown to exert effects on endocrine and immune function in higher vertebrates (Hoffman et al. 2001). Grue et al. (1997) reviewed the sublethal effects of ChE inhibitors on captive small mammals and birds. These include impaired thermoregulation (i.e., pronounced hypothermia, with normal body temperature usually resumed within 24 hours), reduced food consumption and reproductive problems caused by impaired sexual behavior and changes in levels of reproductive hormones (i.e., reduced LH levels). Alterations of thermoregulatory ability have potentially serious consequences to bats due to their metabolic complexities. Gordon (1993) suggests that hypothermia is the natural response

of mammals in order to minimize the effects of toxins such as OPs and CAs. Bats often enter into torpor to conserve energy when demands are high and energy resources are low (Kurta 1990). How anti ChE compounds might affect this poorly understood physiological response is unknown. Reduction in food consumption might also reduce reproductive success, pup or nestling weight and survival of offspring in mammals and birds (Grue et al. 1997; Hill 1995). Reduced reproductive success, leading to less young, would be particularly bad for bats because of their low reproductive rate (most species of bats in Indiana produce only one or two offspring per year, Whitaker and Hamilton 1998). Low reproductive rate dictates slow recovery of affected populations (Clark and Shore 2001). McFarland (1998) analyzed ChE activity in brains of little brown myotis and northern myotis (*Myotis septentrionalis*). Bats were collected from agricultural sites in northern Missouri during summer. As a comparison, bats were collected from cave walls and trapped at cave entrances in non-agricultural areas of southern Missouri on April 17-20 (near the end of hibernation). At this time exposure to insecticide would not be likely. No significant differences in mean ChE activity were evident between the two groups of bats. However, ChE activity in one little brown myotis collected in summer was more than 2 SD below the mean of the comparison group. ChE activity in several other individuals sampled in summer also was low. Based on the presence of brain ChE inhibition in some of the bats, McFarland (1998) suggested that bats may be exposed to sublethal levels of OP and/or CA insecticides applied to agricultural crops in agricultural sites in northern Missouri during summer. O'Shea and Clark (2002) reviewed the potential exposure risks in bats related to OP or CA usage in Missouri and Indiana. Land (2001) was the first to report detections of OP residues in bat guano. Eighteen different OPs were detected in guano from maternity roosts of cave bats (*Myotis velifer*) in Texas, confirming bat exposures to OPs.

Given the mounting evidence that bats in other locations are being exposed to OPs, we implemented 2 small opportunistic studies to determine whether or not Indiana bats might have exposed to OPs, and whether or not OPs might have contributed to mortality in a small subset of bats (Indiana, northern myotis) that had been submitted to the Indiana Department of Health Rabies laboratory over a three year period.

MATERIALS AND METHODS

All bats submitted to the Indiana Department of Health Rabies laboratory since 1966 have been forwarded to JO Whitaker Jr., at Indiana State University for identification and research purposes. Many of these bats exhibited behavior that brought them to the attention of humans, i.e., they were sick, dying, dead or on the ground. From 1966 to 2003, about 200 to 400 bats were submitted annually, but only 5.4% of these proved rabid (Whitaker and Douglas in press).

Nine bats that had been submitted to the Indiana Department of Health Rabies laboratory from 1998 to 2000were submitted through the US Fish and Wildlife Service to the Geochemical & Environmental Research Group, Texas A&M laboratory for chemical analysis in August 2001. Five Indiana myotis and four northern myotis, all non-rabid, were analyzed whole body, minus the brain (which had been removed for rabies testing). Analyses included: OPs, organochlorine insecticides, PCBs, and pyrethroids. Due to the large sample mass required for CA insecticide analysis (35 grams) those contaminants

could not be investigated. The bats were maintained frozen prior to chemical analysis. No stomach content examinations were conducted on these bats.

Recent guano samples were collected from priority hibernacula by placing plastic sheets under roosting / hibernating Indiana myotis. These sheets were retrieved at various intervals and guano pellets were transferred to chemically clean glass jars and frozen until analysis. In addition, we also collected for chemical analysis any fresh dead Indiana myotis specimens found during biennial mid-winter hibernacula surveys.

GC/MS method in SIM mode was used to perform chemical analysis on the carcasses. The tissue samples were extracted by the NOAA Status and Trends Method (MacLeod et al. 1985) with minor revisions (Brooks et al. 1989; Wade et al. 1988). The tissue samples were homogenized with a Teckmar Tissumizer. A 1-10 gram sample (wet weight) was extracted with the Teckmar Tissumizer by adding surrogate standards, Na2SO4, and methylene chloride in a centrifuge tube. The tissue extracts were purified by silica/alumina column chromatography to isolate the aliphatic and PAH/pesticide/PCB fractions. The PAH/pesticide/PCB fraction was further purified by HPLC in order to remove interfering lipids. The quantitative analyses were performed by capillary gas chromatography (CGC) with a flame ionization detector for aliphatic hydrocarbons, CGC with electron capture detector for pesticides and PCB's, and a mass spectrometer detector in the SIM mode for aromatic hydrocarbons (Wade et al. 1988).

RESULTS

OP insecticides were detected in 3 of the 9 bats from the Indiana Department of Health Rabies laboratory (Table 1). Chloropyrifos (0.18 µg/g wet weight) was detected in a northern myotis (JOW no. 16729), submitted on August, 29, 2000. Diazinon (0.034 µg/g wet weight) was detected in a female Indiana myotis (16615), submitted on April, 15, 2000. Methyl parathion (0.015 µg/g wet weight) was detected in a female Indiana myotis (16204), submitted to the rabies laboratory on October, 21, 1998. All the samples contained DDE, dieldrin, oxychlordane and heptachlor epoxide. PCBs were detected in 8 of 9 bats. No pyrethroid insecticides were detected (Table 1). Due to the small size of the sample, no statistical analysis could be performed. In this study, the 3 bats with detectable levels of OP insecticides in their carcasses were found in Vanderburgh County. According to Indiana Agricultural Statistics Service census from February, 1999, 55% of the total land area of Vanderburgh County is farmland (38% corn, 48% soybeans and 12% wheat).

Chlorpyrifos was also detected every Indiana myotis carcass and guano sample from Ray's Cave and Wyandotte Cave, important Indiana myotis hibernacula (Table 2). No other OPs were detected in the Indiana myotis carcasses that were found dead in the caves.

Chloropyrifos is registered in Indiana for use on corn, soybeans and wheat to control agricultural pests and in buildings to control cockroaches, fleas and termites. It is the most popular insecticide used in Indiana. Chlorpyrifos is usually applied to crops or domestic livestock. In 2000, diazinon was registered in Indiana for turf grass applications as well as for a variety of homeowner uses. Preparations used in agriculture and by exterminators are in liquid form and contain 85–90% diazinon. Diazinon preparations available for home and garden use contain 1–5% diazinon as liquid or granules although

residential uses are being phased out. In 1998, methyl parathion was registered for a variety of agricultural products such as corn, soybeans and wheat. Methyl parathion should only be used in open fields to control insects and is used as a spray. Indiana does not require submission of pesticide use records (present insecticides data courtesy of G. N. Saxton, Compliance Officer, Office of Indiana State Chemist, 2005 and ATSDR, Agency for Toxic Substances and Disease Registry).

Diclorvos was in Indiana myotis guano from 3 of the 4 caves at concentrations ranging from 11 – 86 ppb, wet weight (Table 2). Dichlorvos is an OP insecticide registered for indoor, indoor food (i.e., mushrooms), terrestrial non-food outdoor, and domestic outdoor use, including such areas as turf, ornamental plants, recreational areas, trails, outdoor living areas, eating areas of drive-in restaurants, refuse areas, garbage collection/disposal areas, loading docks, animal feedlots, stockyards, and corrals (US Environmental Protection Agency undated). Its target pests include flying or resting adult mosquitoes, flies, gnats, chiggers, ticks, cockroaches and other nuisance insect pests (US Environmental Protection Agency 2000). This is the second confirmation that diclorvos does enter the food chain and provides a dietary exposure route to bats as did Land (2001).

DISCUSSION

Improvements in Analytical Capability

Even though this data is limited in scope, it raises questions regarding the lack of understanding anti-ChE insecticide toxicity, especially in bats. Conventional wisdom suggests that there is little bioaccumulation of OPs in living tissues because OPs are not particularly fat soluble, and several authors have suggested that the presence of OPs is indicative of recent exposure prior to death (O'Shea and Clark 2002; Hill 1995; Hill 1989). Although this may be true in some cases, the presence of OP residues in the absence of demonstrated ChE inhibition, does not confirm clinical affects nor suggest causation in a specific mortality.

Clearly there has been recent improvement in OP analytical capabilities. Perhaps what once could only be detected when approaching lethal doses can now be detected at substantially lower levels. Chlorpyrifos was detected (0.01 ppm the detection limit) in cream from cows fed a diet containing 3 to 10 ppm after 3 weeks of dosing (McKellar et al. 1976), but cream residue levels never exceeded 0.1 ppm nor was it detected 3 days after dosing ceased. Schmidt et al. (2002) collected 74 bats (mainly eastern red bats, *Lasiurus borealis* and eastern pipistrelles, *Pipistrellus subflavus*), using mist nets, from 11 OPs exposure sites in Missouri during spring and summer 1997-1998. No OPs were detected in any of the bats sampled. Schmidt et al. (2002) states "Chlorpyrifos and malathion were not detected in bats collected on FLW or at reference sites, but this was somewhat expected. Organophosphorous pesticides are generally hydrolyzed and they do not bioaccumulate in mammals or concentrate in food chains (Hoffman et al. 1995)."

Yet, in studies done in the following 2 field seasons by these same researchers, chlorpyrifos was detected at low levels in nearly every bat carcass analyzed (BHE 2004, BHE 2005). In fact, chlorpyrifos has been added to the routine analyte list for organochlorine pesticides because it is now commonly found in tissue samples – its analytical half life is estimated to be approximately half of that of DDT (pers. comm.,

Terry Wade, Analytical Chemist, GERG, Texas A&M University, November 14, 2005). Land (2001) clearly demonstrated OPs are entering the food chain, as evidenced the presence of dichlorvos in cave myotis guano. If we can assume negligible bioaccumulation in bats, our dichlorvos guano results may approximate ambient dosing levels near these hibernacula, although the "shelf life" of OPs in guano is also unknown.

Lack of Toxicity Data

Only two laboratory studies using single dose, acute toxicity studies exist using OPs and bats (Clark 1986, Clark and Rattner 1987). Because acute toxicity tests deal with high doses and looked primarily at death in 24 hours as the measurable endpoint, their relevance to field conditions and chronic exposures is lacking. Clark (1986) dosed little brown myotis and big brown bats (*Eptesicus fuscus*) by stomach intubation with methyl parathion. He found that bats lost coordination within 1 hour and were still unable to right themselves at 24 hours after exposure. Although it appears that bats are 8 times less sensitive than mice in terms of acute lethality, the oral dose estimated to cause loss of coordination in 50% of big brown bats was one-third or less than the LD50 of this species. Clark and Rattner (1987) tested acephate in a similar fashion and also found a lack of acute toxicity. On the one hand, this implies that exposure to non-lethal doses could result in death in the field when bats become debilitated and if they are exposed to high risk situations such as predators, harsh sun, inclement weather, or drowning (Clark 1986). Even though ChE inhibiting insecticides are labile in mammals; recovery of inhibited ChE activity from a single exposure may vary from a few hours for most CAs to 1-3 weeks for OP compounds (Hill 1995). Repeated application of even short-lived chemicals may cause cumulative physiological effects without a corresponding accumulation of chemical residues (Hill 1995). A more significant question was whether or not the lack of lethality in during the 24 hour studies might be related to dosing that began as the bats were entering their inactive period of the day and potentially their ability to enter torpor when subjected to stress, in this particular case, the stress of the toxicity study (Gordon 1993). This could have delayed acute effects and making it appear that bats are not as "sensitive" to OPs as mice. Energetic requirements, daily patterns of activity and other behaviors of vertebrates also may be affected (Hill 1995). Alterations of thermoregulatory ability have potentially serious consequences to bats and how anti-ChE compounds might affect in this manner is unknown.

Diagnosis of mortality in bats due to exposure to OP or CA insecticides is difficult. Control groups are necessary for comparison of ChE activity levels and confirmation of the presence of insecticidal compounds in digestive tracts or whole body analysis is desirable. Nonetheless, there are likely scenarios for such effects. Exposure during foraging or traveling may be respiratory or dermal, and therefore no residues would be detected in the contents of the digestive system (O'Shea and Clark 2002). Clark (1986) has demonstrated that depression of ChE activity in brains of little brown myotis was similar whether dosage was oral or dermal. Grue et al. (1997) states that recovery of ChE activity levels in vertebrates that survive exposure to OPs occur primarily by synthesis of new, uninhibited ChE enzyme. The rate of recovery of brain ChE appears to vary among chemicals and depends on maximum level of enzyme inhibition. Initial recovery of enzyme inhibition to 50%-60% of normal is rapid and more gradual thereafter, with recovery to normal levels in up to 30 days after exposure ceases and long after all residues are eliminated. Inhibition of ChE by s is reversible with decarbamylation and recovery occurring within a few hours for most CAs (Grue et al. 1997; Hill 1995). Bats

with lowered ChE activity could be subject to sublethal effects such as loss of coordination, impaired echolocation and elongated response time that might contribute to eventual mortality (O'Shea and Clark 2002). Being effected while foraging and away from the roost makes those bats more likely to be found by predators and not diagnosed as effected by insecticides especially since most studies conducted around roosts.

Even determination of ChE activity in brains can be complicated. Postmortem reactivation of ChE can hamper ability to confirm effects of ChE inhibiting substances in bats that have died at unknown times prior to collection (O'Shea and Clark 2002; Hill 1989). Clark (1986) demonstrated that bats and mice heavily dosed with the OP methyl parathion showed less ChE depression than the less heavily dosed ones. He suggested that the heavily dosed animals died quickly, before there was sufficient time for the brain cholinesterase activity to decline. It is important to state that in the present study the cause of death was not determined nor was it determined how long the OP infected bats lived after they were exposed. It is also important to state that the samples were in the freezer for 10 months to 3 years prior to the chemical analysis. Given that OPs are supposedly rapidly eliminated from the living body (Hill 1989) and do not persist in the environment, it is likely that the residue levels detected are lower than were in the body at the time of exposure.

(Maul and Farris 2005) documented significant levels of cholinesterase (ChE) inhibition in 8.7 percent of northern cardinals (*Cardinalis cardinalis*) sampled from agricultural field edges in northeast Arkansas, from 3 to 21 days post treatments. If sublethal reductions are occurring in the ChE enzymes of Indiana bats as a result of these documented OP exposures, and if ChE reductions are cumulative, persisting for many days, both foraging ability could be impaired such that their high energy requirements could not be met and increasing the risk of trauma due to navigational impairment. Gullien et al. (1991) documented whole body residues of fenitrothion in *Pipistrellus pipistrellus* the day after (0.54 ppm) and 21 days after (0.84 ppm) rice fields were treated. In addition, mean brain AChE levels in bats collected 21 days after treatment were significantly reduced.

More research needs to be done on the sublethal effects of this widely used class of pesticides.

Questions Raised by Residues in Dead and Dying Bats

The fact that OPs residues were found in carcasses in Indiana indicate exposure to those pesticides. Often bats necropsied at the U.S. Geological Service Wildlife Health Laboratory in Madison are found to be emaciated (Grace McLaughlin, USGS Wildlife Health Laboratory, pers. comm.). Cause of death could be related to adverse weather conditions such as a drought-induced lack of prey or an unexpected cold snap that also eliminating food availability for too long. Alternatively, a sublethal dose of an organophosphate or could perhaps reduce a bat's foraging capability for a few hours or days and that could be sufficient to cause starvation related mortality that would be nearly untraceable using current analytical chemistry techniques.

Although it is possible that the bats were exposed to OPs by dermal or respiratory routes while flying in areas where an OP had been sprayed, it is most likely that these OP residues come from consuming contaminated insects. It is likely that the diazinon-

exposed Indiana myotis resulted from insects associated with a spring-time turf grass application. Just emerging from hibernation therefore having low energetic reserves could have made that bat more sensitive to the toxic effect of OPs. It is possible that bat number 16729 (northern myotis) was exposed to chloropyrifos due to agricultural application or other use in August. It is more difficult to suggest a reason for using methyl parathion at the end of October, but bat number 16204 (Indiana myotis) was found in October containing residues of this OP insecticide. According to Hill (1989) if this toxicant does not rapidly bind with target enzymes in blood or nerve tissues, it is soon metabolized and excreted. If this is so, then it is likely that exposure occurred shortly before death.

Indiana myotis (16204) submitted in October had detectible levels of methyl parathion and a whole body lipid concentration of 33.7%. Speakman and Thomas (2003) indicate 30% fat reserves as the normal level prior to hibernation. Northern myotis (16729) submitted in August and found with detectible levels of chloropyrifos appeared to be in good condition, based on 15% lipid concentration in its body. Bats just emerging from hibernation have relatively low body weight and low lipid concentration in their bodies. Fenton (1988) stated that by the end of hibernation, bats have lost up to 25% of the weight they had the previous autumn, and their fat reserves are severely depleted. In April, Indiana myotis (16615), contained detectible levels of diazinon, and had a normal lipid concentration of 3.5%. Although the cause of death was not determined in any of the 9 bats sampled, it is possible that OP insecticide exposure was a contributing cause of death in these 3 three bats, especially since all three were in good nutritional status.

Of the 8,262 bats submitted to the Indiana department of health rabies laboratory during the years 1966-2003, only 5.4% (445 bats) were rabid (Whitaker and Douglas in press). This means that more than 94% of the bats died from other causes. Considering that many of these bats were found under suspicious circumstances (.i.e. often ending up on the ground around human habitats), and that OPs can adversely influence behavior, we suggest that OPs may have directly or indirectly led to the deaths of the 3 bats. Based on these findings, we also expect to find similar exposure rates if additional bats are tested. The fact that 3 of 9 bats that were selected out of the population of sick or dead bats were found to be exposed to OPs might suggest important exposures to those toxicants in the overall population. Because of this, and also because of the lack of information regarding the sublethal effects of these ubiquitous neurotoxins to living bats, more research is needed.

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Table 1. Organophosphate and organochlorine insecticides (µg/g wet weight) in Indiana bats and northern long-ear bats found by members of the public and submitted to Indiana Department of Health for Rabies testing.

	Myotis sodalis					Myotis septentrionalis			
ID	*16122*	*16204*	*16615*	*16638*	*16639*	*16729*	*16730*	*16784*	*16785*
Date Found	8/26/1998	10/21/1998	4/15/2000	8/22/2000	8/23/2000	8/29/2000	8/29/2000	10/6/2000	10/18/2000
County	Marion	Vanderburgh	Vanderburgh	Vanderburgh	Marion	Vanderburgh	Decatur	Davies	Vanderburgh
Body mass (g)	5.44	7.4	4.73	5.8	6.08	4.94	4.03	6.39	6.12
Sex	M	F	F	U*	U*	U*	U*	M	M
% lipids	12.9	33.7	3.5	9.1	3.0	15.0	3.5	22.8	40.3
% moisture	60.7	40.2	56.5	62.2	61.5	49.8	51.4	51.6	28.8
Dieldrin	0.06	0.13	0.09	0.09	0.08	0.10	0.04	0.16	0.23
Heptachlor epoxide	0.02	0.14	0.02	0.02	0.01	0.02	0.01	0.11	0.05
Oxychlordane	0.1	0.19	0.14	0.21	0.09	0.21	0.04	0.12	0.28
p', p'-DDE	0.12	0.14	0.3	0.14	0.14	0.16	0.03	0.07	0.06
PCB-total	0.62	0.53	1.3	0.92	--	4.2	0.89	0.39	0.8
Organophosphates:									
Chlorpyrifos	--	--	--	--	--	0.18	--	--	--
Diazinon	--	--	0.03	--	--	--	--	--	--
Methyl parathion	--	0.02	--	--	--	--	--	--	--

* U, sex was not determined.

Table 2. Organophosphorous insecticide residues (ppb, wet weight) in Indiana myotis guano and carcasses from several priority Indiana myotis hibernacula in Indiana.

	Guano from Caves					whole *Myotis sodalis*					
						Rays Cave		Wyandotte Cave			
	Coon	Grotto	Rays	Rays	Wyandotte						
Date	4/2004	4/2004	4/2004	9/2003	4/2004	1/2003	4/2004	2/2002	2/2003	2/2003	2/2003
Mass (g)						7.9	8.0	6.2	4.6	5.1	5.9
Lipid	0.4	0.1	0.3	0.7	1.1						
Sex						F	U*	F	M	M	M
Moisture	51.8	69.7	67.2	76.2	13.5						
chlorpyrifos	<0.1	<0.08	0.7	0.2	1.0	0.8	1.1	1.6	3.2	4.2	1.1
dichlorvos	86	11	--	--	20						

OPs that were not detected include: dimethoate, disulfoton, ethoprop, fensulfothion, fenthion, malathion, merphos, methyl parathion, EPN, azinphos-methyl, phorate, trichloronate, coumaphos, demeton, diazinon, tetraethylpyrophosphate, mevinphos, monocrotophos, naled, parathion, ronnel, sulfotepp, sulprofos, tetrachlorvinphos, and protothiofos.

* U, sex was not determined.