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Variation in plasma cholinesterase activity in the clay-colored robin (*Turdus grayi*) in relation to time of day, season, and diazinon exposure

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Abstract Cholinesterase (ChE) activity in birds is subject to interspecific and intraspecific species variations. Factors that influence enzyme activity have to be taken into account in order to obtain an accurate estimation of cholinesterase inhibition due to pesticide exposure in wild birds. This study evaluates variation of plasma cholinesterase activity in clay-colored robin (*Turdus grayi*) in relation to time of day, season, and exposure to diazinon. Other variables that can affect cholinesterase activity such as weight are also taken into account. The birds were marked, weighed and sexed using the cloacal technique. One dose of commercial diazinon mixed with papaya was fed to each bird at concentrations of 0.0, 0.5, 1.5 and 3.0 mg/kg ai. The results showed differences in ChE activity between seasons ($t = -3.07$, $P < 0.05$). Also, diurnal plasma cholinesterase variations were observed (20% in 2 h). The highest inhibition values were 73% for birds dosed with 1.5 mg/kg ai. Our study provides field and laboratory data on variation of ChE activity in a tropical bird species. Knowledge of the variation of ChE in the clay-colored robin will enable us to use this species as an indicator of exposure to ChE inhibiting pesticides in tropical agroecosystems.

Keywords Cholinesterase inhibition · Clay-colored robin · Diazinon · Organophosphorous insecticides · *Turdus grayi*

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Introduction

Inhibition of cholinesterase (ChE) activity in birds is used as an indicator of exposure and poisoning by organophosphorous pesticides (Thompson 1999). There is interspecific and intraspecific variation in ChE activity in birds; however, no relationships between such variations have been determined (Busby and White 1991; Thompson and Walker 1994). In birds, the female generally shows higher enzyme activity than the male (Hill 1989), with exception of some species such as the great tit (*Parus major)* (Cordi et al. 1997). Furthermore, it has been observed that ChE activity varies with age (Fleming 1981; Gard and Hooper 1993; Wolfe and Kendall 1998) and with weight (Rattner and Fairbrother 1991; Cordi et al. 1997). Diurnal variation of ChE activity also occurs in various bird species and is characterized by a gradual reduction in activity as the day proceeds, eventually returning to its base value (Thompson et al. 1988).

Ludke et al. (1975) suggested that more than 50% brain cholinesterase inhibition is sufficient to diagnose death as a result of organophosphorous pesticide poisoning. It is also considered that more than 20% inhibition indicates exposure to these substances (Ludke et al. 1975). Lari et al. (1994) exposed Japanese quail (*Coturnix coturnix* *japonica*) to methyl-azinphos and found that plasma butyryl cholinesterase (BChE) displays a linear correlation with brain cholinesterase and that the toxicity values proposed by Ludke et al. (1975) would be equivalent to 75% BChE inhibition for a diagnosis of death by pesticide poisoning and 30% BChE inhibition for pesticide exposure.

Birds exposed to organophosphorous pesticides (OPs) can reach maximum inhibition of cholinesterase activity in a few hours, then completely recover in 4–5 days. Birds are mainly poisoned through their food and through dermal exposure. The pesticide type determines the level of toxicity; thus, parathion is more toxic than diazinon which in turn is more toxic than malathion (Hooper et al. 1989). However, two different species with the same feeding habits can present different inhibition percentages, probably due to their respective physiological abilities in eliminating the pesticide (Neithammer and Basket 1983). Positive correlations between enzyme activity values and bird weight have been observed (Cordi et al. 1997; Cobos-Gasca et al. 2006); thus, it is important to take body weight into account when comparing enzyme activity values between exposed and non-exposed individuals. Organophosphorous insecticides are commonly used in agriculture in the Yucatán Peninsula and diazinon is the most utilized, particularly in papaya plantations and in horticulture in general (Alvarado et al. 1994). Approximately 80% of the insecticides used in the region are OPs, the rest are organochlorines, carbamates, and pyrethroids (Alvarado et al. 1994). Organophosphorous compounds are applied to papaya and vegetable crops two to three times a week during the growing season in spring and summer, although papaya grows all year round, therefore, applications to papaya plantations are more continuous. This study evaluates diurnal and seasonal (two seasons) variation of plasma cholinesterase in the clay-colored robin (*Turdus grayi*) exposed and non-exposed to diazinon. The clay-colored robin is an abundant species, widely distributed throughout the Yucatan Peninsula; it is an omnivore and can be maintained in captivity. During spring and summer robins feed primarily on fruits and during the winter they switch to insects because fruits are more scarce (personal observation). The aim of the study was to obtain baseline data on ChE activity in the clay-colored robin to test its suitability as a bioindicator for organophosphorous pesticide exposure in tropical agroecosystems. The influence of weight on cholinesterase activity is also examined.

Materials and methods

Twenty clay-colored robins were captured during each season in spring (March) and autumn (November), 2005. The birds were captured with traps in areas of little agricultural activity in Yucatan, near the town of Hunucma (21°01′ N, 89°52′ W). The birds were weighed using a digital balance (±0.1 g), and sexed using the cloacal technique (Ralph et al. 1995). Groups of five birds were placed in cages for a period of 2 weeks and fed a diet of fruit and balanced food. Subsequently, two 75 μl capillary tubes of blood were taken from each bird at the same hour (8.00 a.m.) by puncturing the brachial vein. The blood samples were kept in refrigeration for less than 24 h until further analysis. Bird captures were conducted under approved permits from the Mexican federal government agency, SEMARNAT.

To study the diurnal variation displayed by enzyme activity (experiment 1), five clay-colored robins were randomly selected and placed in individual cages. After a week of acclimatization, two 75 μl blood samples were taken from the brachial vein of each bird every 2 h, from 8:00 until 14:00 hours for 1 day. This short period was selected to avoid injuring the birds' veins as result of the continuous blood sampling.

To evaluate enzyme activity inhibition due to diazinon ingestion (experiment 2), 20 clay-colored robins were randomly selected and placed in four cages, each containing five individuals. All the birds were kept under the same environmental and feeding conditions. One blood sample was taken prior to the experiment to determine the basal ChE activity level. One dose of commercial diazinon ("Dragón", 25% diazinon active ingredient, ai) mixed with papaya was fed to each bird using a glass syringe. The doses given were selected after considering the mean value of LD_{50} estimated for diazinon in birds (5.25 mg/kg, Mineau et al. 2001). The doses were 0.0 (control group), 0.5, 1.5 and 3 mg of diazinon (ai) per kilogram of body mass. To ensure that the dose was not regurgitated with the papaya, the birds were observed for a period of 15 min immediately following dosing. After 24 h, a duplicate set of blood samples were taken from the brachial vein and the level of plasma cholinesterase activity was determined. A blood sample was taken daily from each bird at the same hour until the cholinesterase levels were restored to their base value. Handling and dosing of wild birds was conducted following University of Yucatan guidelines for the proper use of animals in research.

Only male robins were used in the experiments as these were captured in greater numbers. The plasma cholinesterase diurnal variation and inhibition experiments were all performed on birds captured during November.

Esterase assays

The samples of blood obtained in each of the experiments were centrifuged at 1000 rpm separating the plasma which was drawn off and stored at −4°C. The evaluation of plasma cholinesterase (ChE) activity was carried out the following day with the Ellman et al. (1961) method modified by Hill and Fleming (1982). This method is based on enzyme reactions whereby cholinesterase is hydrolyzed by acetylthiocholine forming thiocholine and acetate. The thiocholine reacts with the dithiodinitrobenzoic acid (DTNB) forming thionitrobenzoic acid which is yellow colored and can be read spectrophotometrically (at 450 nm). The range of colours produced indicate cholinesterase activity. The sample analysis was undertaken at ambient temperature (25°C).

Statistical analysis

Analysis of covariance (ANCOVA) was applied to the data obtained from the first experiment (weight and ChE activity) to determine the effect of weight on the relationship between ChE activity and time. If weight did not have an effect, then an analysis of variance (ANOVA) of randomized blocks (individuals) was applied to determine if there were differences in plasma cholinesterase levels due to the effect of time. For each of the doses applied in the second experiment we calculated the mean percentage value of inhibition on enzyme activity, with respect to the control. These values were then compared using Duncan's multiple comparison test. Friedman's repeated measures analysis of variance on ranks was calculated to test ChE activity at different schedules (08:00, 10:00, 12:00, and 14:00 hours). All the statistical tests were carried with the use of STATISTICA (Statsoft 1996). The level of significance was set at $P < 0.05$.

Results

The analysis of covariance indicated that weight did not have an effect on cholinesterase activity during both seasons ($F_{1,13} = 0.31$, $P > 0.05$). Without the effects of weight there were significant differences in ChE activity between seasons ($t = -3.07$, $P < 0.05$; Fig. 1); with higher values during autumn than during spring (average 35%).

The results of the Friedman's repeated measures analysis of variance on ranks indicate that there were no significant differences in median plasma cholinesterase activity values among the different collection schedules from 8 to 14 h ($P = 0.075$). However, there is a descendent trend of cholinesterase values during the day, being high during the early hours of the day and later they fell to 30% (Fig. 2).

Higher doses of diazinon resulted in higher inhibition (Table 1). The highest inhibition values were 73% for the 1.5 mg/kg ai dose and 64% for the 0.5 mg/kg ai dose. The

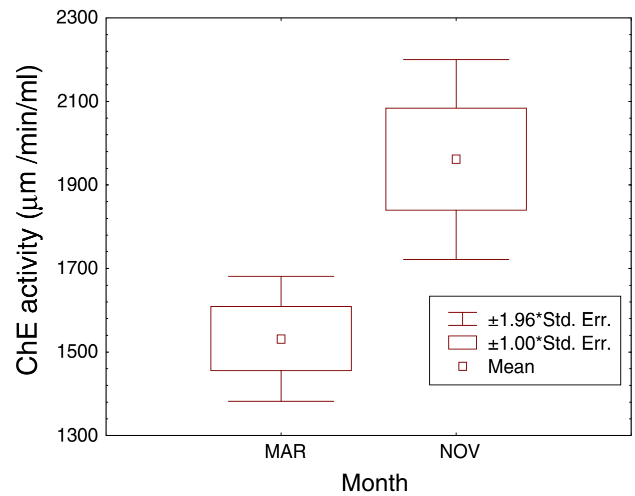

Fig. 1 Seasonal variation of plasma cholinesterase activity in the clay-colored robin

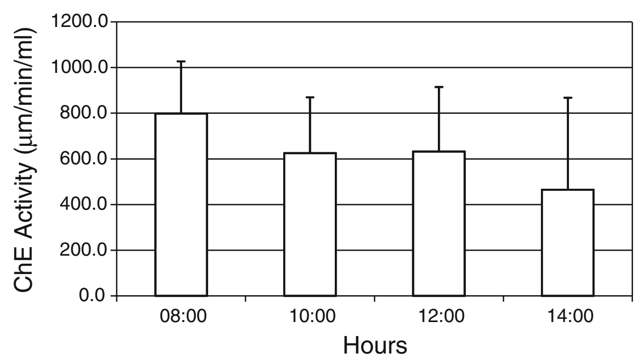

Fig. 2 Diurnal variation of plasma cholinesterase activity in the clay-colored robin

recovery of plasma cholinesterase on the second day after dosing was faster when the dose was low, resulting in a complete recovery by the fifth day. Poisoning effects on birds exposed to a dose of 1.5 mg/kg included trembling, panting and little movement. However, the following day after exposure behavior of birds was normal. All the individuals exposed to a dose of 3 mg/kg died during the 2 h period after ingestion, displaying trembling, paralysis and fecal deposits.

Discussion

Seasonal changes in ChE activity have been reported in a wide range of birds (Hill and Murray 1987; Thompson 1999). Plasma ChE activity was 8% lower in the great tit during the fall than in the spring (Norte et al. 2009), which is opposite to what we observed in the robin. Rattner and Fairbrother (1991) reported a 15% difference in brain ChE between winter and spring in the red winged blackbird

Table 1 Plasma cholinesterase (ChE) inhibition in the clay-colored robin exposed to diazinon

Exposure days	*n*	0.5 (mg/kg)		1.5 (mg/kg)	
		% inhibition (mean ± SD)	Observed effect	% inhibition (mean ± SD)	Observed effect
1	5	67.28 ± 7.14	Normal activity	73.90 ± 3.25	Tremors, panting and little movement
2	5	47.00 ± 11.16	Normal activity	68.35 ± 1.48	Normal activity
3	5	37.83 ± 3.23	Normal activity	53.35 ± 18.74	Normal activity
4	5	24.33 ± 9.42	Normal activity	30.30 ± 16.83	Normal activity

(*Agelaius phoeniceus*); although it is not specified in which season the activity was higher or lower. The difference in ChE activity in blackbirds is lower than the 35% difference observed in our study. A similar pattern was observed in the plasma cholinesterase activity of the northern bobwhite (*Colinus virginianus*) but not in the European starling (*Sturnus vulgaris*), common grackle (*Quiscalus quiscula*) and red-winged blackbird (Rattner and Fairbrother 1991). Fildes et al. (2009) also recently reported a significant difference in plasma ChE activity between summer and winter in zebra finches (*Taeniopygia guttata*). The above emphasizes the need to obtain control values from birds captured during the same season when conducting field studies of cholinesterase inhibition in birds.

The observed differences in ChE activity of the clay-colored robin during fall and spring could be a result of changes in feeding habits (Chable-Santos et al. 2007; Llamosa 2008). In spring, the robin can be observed foraging in a variety of fruit trees, particularly papaya crops during the early hours of the day. We assumed that most of the insecticide exposure in robins probably occurs through the ingestion of papaya since this fruit is sprayed constantly throughout the year. During autumn, the robin forages on worms in the muddy soil formed by rainwater, probably due to the rainy season and the scarcity of fruit (personal observation).

Diurnal variation of cholinesterase has been observed in birds. Changes in BChE and ChE serum activity have been reported for buzzards (*Buteo buteo*) and European starlings, the latter with an increase of 150% in ChE serum and a non-significant variation in BChE serum (Gard and Hooper 1993; Garcia-Rodriguez et al. 1987). In buzzards, an increase of 50% in serum ChE was observed (Garcia-Rodriguez et al. 1987). Our results for the clay-colored robin (30% decrease) coincide in part with those observed in northern bobwhite hens and in tree sparrows (*Passer montanus*) whose cholinesterase values decreased 22 and 35% respectively during the day (Rattner and Fairbrother 1991; Thompson 1991). However, the variation observed in the clay-colored robin corresponded to a shorter day period (8–14 h) than the observed in other studies, which was longer. This decrease in serum ChE puts the bird's health at risk because, if exposed to pesticides, the enzyme activity would be drastically inhibited. In contrast, an increase in enzyme activity during the day would provide the birds with greater resistance to intoxication.

In many bird species the highest AChE inhibition values have been observed 24 h after the administration of the pesticide dose (Zinkl et al. 1984). However, more than 80% inhibition of serum cholinesterase has been observed in Japanese quail, 7 h after exposure to a 50 mg/kg dose of methyl-azinphos (Lari et al. 1994). In starlings, an 80% inhibition of serum cholinesterase was observed 3 h after exposure to 3 mg/kg of demethon-*S*-methyl (Parker and Goldstein 2000). The inhibition value (73%) obtained in our study 24 h after exposure with diazinon, is lower than that obtained by Fossi et al. (1994) when exposing the Japanese quail to a 10 mg/kg dose of azamethiphos. It is well established that birds' sensitivity to anticholinesterase exposure is quite variable (Hill 1992). Our results support previous observations that the inhibition produced by an insecticide depends on the type, the dose, and the species to which it is administered (Hooper et al. 1989; Neithammer and Basket 1983).

Diazinon is one of the insecticides more frequently associated with avian mortality incidents in the United States (Fleschli et al. 2004). The American Robin, a relative of the clay-colored robin, is one of the species that has been most commonly affected. Rondeau and Desgranges (1995) found 48% ChE inhibition in blood serum of American robins from christmas tree plantations which had been sprayed with diazinon at 0.625 kg ai/ha. Also, ChE inhibition up to 72% was reported in the same species due to exposure to 2.2 kg ai/ha diazinon on ornamental trees (Decaire et al. 1993). It would be important to determine if the clay-colored robin is as sensitive to diazinon exposure as the American robin. We need to learn more about other factors which are also likely to affect ChE activity in the clay-colored robin, so that the full impacts from exposure to OPs while foraging on fruit crops in the Yucatan Peninsula can be established.

Among some reasons that are used for explaining interspecific variation in plasma ChE activity in birds, include metabolism (associated with body mass), phylogeny, and food sources. Studies of plasma ChE activity in related species have shown that there is a strong relationship between ChE activity and phylogenetic classification (Westlake et al. 1983; Claudie et al. 2005). Strum et al. (2008) reported that plasma BChE activity decreased with increasing body mass in shorebirds; and, Fossi et al. (1996) reported that some omnivorous bird species had higher plasma ChE activities than other species with a narrow diet.

Our study provides data that support the use of the clay-colored robin as an indicator species of contamination by organophosphorus insecticides in tropical agroecosystems, particularly in fruit plantations where the bird forages. Very little is known about the impacts of OPs and carbamate insecticides on birds foraging in fruit growing areas in tropical environments.

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