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SEX AND STORAGE AFFECT CHOLINESTERASE ACTIVITY IN BLOOD PLASMA OF JAPANESE QUAIL

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ABSTRACT: Blood plasma cholinesterase (ChE) activity is a sensitive indicator of exposure to organophosphorus and carbamate insecticides. Effects of sex and storage of samples were studied as sources of variability by treating breeding Japanese quail (*Coturnix japonica*) with 3 mg of dicrotophos or carbofuran per kg of body weight and comparing blood plasma ChE activities for samples collected at 1 hr postdosage and assayed fresh, after 1 and 2 days of refrigeration (4 C), and after 1, 7 and 28 days of freezing(−25 C). ChE activity of fresh control plasma was 34% ($P < 0.01$) higher in males than females. Male ChE activity remained essentially unchanged during storage while female ChE activity increased ($P < 0.05$) gradually over time under both storage conditions. In contrast, when plasma ChE activity was inhibited by either antiChE, male plasma ChE activity was depressed further than female ChE ($P < 0.01$) and remained constant during storage while female ChE activity continued to decrease ($P < 0.05$). These divergent effects of exposure to antiChE compounds and sample storage indicate extreme care should be exercised in use of blood plasma for evaluation of antiChE exposure in wild birds.

Key words: Japanese quail, *Coturnix japonica*, plasma cholinesterase, carbofuran, dicrotophos, clinical method, storage, freezing, refrigeration.

INTRODUCTION

Brain cholinesterase (ChE) activity is routinely measured in free-living animals to indicate exposure to organophosphorus or carbamate pesticides. Blood erythrocyte and plasma ChE activity has long been used to monitor occupational exposure of workers to antiChE compounds. Brain is usually the tissue of choice for wildlife studies because inhibition of this source of ChE can be correlated with certain acute responses (Decandole et al., 1953; Ludke et al., 1975) and common species can be sampled without excessive population effect; whereas, the biological function of blood ChE's is not clear (Kutty, 1980). However, blood may actually be the better source of ChE for simple detection of antiChE presence in a species habitat, and its inhibition indicates recent exposure. Plasma ChE may be inhibited as much as 75% when brain ChE inhibition reaches a statistically detectable 10 to 20% (Ludke et al., 1975), and plasma ChE returns to normal within 1 to 2 days (Westlake et al., 1981a, b) while brain ChE takes 1 to 3 wk to recover (Fleming and Grue, 1981). Use of blood ChE is nondestructive and permits repeated sampling of the same individual and sampling where study design or population status precludes specimen removal. This study addressed the effects of several variables, including sex, refrigeration, and freezing, that may influence use of plasma ChE in field research.

MATERIALS AND METHODS

Sixty 10- to 12-wk-old Japanese quail (*Coturnix japonica*) were selected from a randomly bred but genetically closed colony maintained at the Patuxent Wildlife Research Center (Laurel, Maryland 20708, USA). Ten birds per sex were assigned by random numbers into each of three galvanized mesh brood units (70 × 100 × 24 cm high) and allowed free access to tap water and unmedicated gamebird breeder diet (Ziegler Brothers, Inc., Gardners, Pennsylvania 17324, USA). The light regime and ambient temperature of 14 L:10 D and 24 to 26 C were the same as when in colony. After 1 wk of conditioning, feed was withdrawn from all pens at 1600 hr and the birds were fasted overnight.

The next morning (0800 to 0900 hr), all birds (10 per sex per treatment) received an equivalent oral dosage of either corn oil (control) or corn oil containing antiChE at a level expected to significantly inhibit brain ChE in all subjects but kill only a few (e.g., <30%). Birds were then observed for about 60 min and survivors were bled by heart puncture and euthanized with CO_2. The treatments were either 3 mg/kg body weight (BW) of technical grade dicrotophos (phosphoric acid 3-(dimethylamino)-1-methyl-

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3-oxo-1-propenyl dimethyl ester; 85% AI) or carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranol methylcarbamate; 99% AI) dissolved in corn oil (W/V) or pure corn oil administered at a final rate of 5 μl/g BW. Blood samples of eight birds per sex (the least number of survivors) and treatment were drawn into 4 ml ammonium heparinized syringes, chilled in wet ice, and then centrifuged at 2,200 *g* for 10 min. Subsamples (100 μl) of each plasma were aspirated into capped plastic vials and assigned by random numbers to immediate ChE assay control ($n = 2$) or storage at 4 C ($n = 4$) or −25 C ($n = 6$). Brains of dead birds were promptly prepared in chilled buffer for same day whole brain ChE assay. Plasma and brains were processed as previously described (Hill and Fleming, 1982) and ChE assays were performed spectrophotometrically with a double-beam spectrophotometer at 405 nm and 25 C (Ellman et al., 1961). Acetylthiocholine iodide (ASChI) was used for substrate.

The two randomly assigned subsamples (i.e., fresh) of each plasma were continued on wet ice until assayed for total ASChI-responsive ChE (primarily butyrylcholinesterase, EC 3.1.1.8, but including other unspecified esterases) between 1100 and 1300 hr on the day of dosage. The duplicate subsamples were averaged and the mean used to represent the ChE activity for the specimen at its death. Two subsamples of each refrigerated (4 C) plasma were randomly selected for assay between 1100 and 1300 hr at 1 and 2 days posttreatment. Likewise, two frozen (−25 C) subsamples of each plasma were selected, thawed and agitated in a water bath (25 C), and assayed between 1100 and 1300 hr on days 1, 7 and 28. Brain ChE (primarily acetylcholinesterase, EC 3.1.1.7) was assayed fresh to establish the relationship between the two enzyme sources at euthanasia. Treatments were analyzed for sex and storage effects on ChE activity by two-way analysis of variance and means were separated by Tukey's HSD procedure. Statistical significance for *F*-tests and mean separations was set at $\alpha = 0.05$. ChE activity of an individual was considered inhibited if it was more than two standard deviations below the arithmetic mean for the control group (Copeland, 1974; Hill and Fleming, 1982).

RESULTS

All quail appeared healthy and in good flesh when selected for dosage and were determined by postmortem examination to have been in reproductive condition. Females were significantly ($P < 0.01$) heavier than males in all study groups as average weights within the treatment groups varied from 141 (SD = 12.5) to 146 g (4.9) for females and 122 (10.4) to 127 g (9.4) for males. Although females averaged 14% heavier than males and appeared to have more visceral and subcutaneous fat, differences were not detected between sexes in their overt responses to the potentially lethal dosage of 3 mg/kg BW of either dicrotophos or carbofuran.

TABLE 1. Brain cholinesterase activity[a] in Japanese quail orally dosed with 3 mg of dicrotophos or carbofuran per kg of body weight and euthanized with CO_2 approximately 1 hr posttreatment.

Sex	Mean	SE	Minimum, maximum values
Control			
Male	15.6	0.38	13.6, 17.3
Female	15.1	0.32	13.9, 16.1
Dicrotophos			
Male	10.5	0.87	6.4, 12.5
Female	10.2	0.99	6.9, 13.3
Carbofuran			
Male	6.7	1.39	3.3, 11.2
Female	6.8	1.17	3.4, 10.4

[a] Cholinesterase activity is μmol acetylthiocholine iodide hydrolyzed/g (wet wt) of whole brain tissue at 25 C ($n = 8$ per data point). All dicrotophos and carbofuran means are significantly less than their respective controls ($P < 0.01$).

Quail dosed with dicrotophos began to experience wing and tail tremors within 10 to 15 min and most became sedentary, ataxic and tended to huddle together after about 30 min. At time of euthanasia, most birds appeared reasonably stable although overtly sick and one male and two females died. Brain ChE activity of the survivors was depressed ($P < 0.05$) by averages of 32 and 33% in females and males (Table 1). Significant ($P < 0.05$) brain ChE inhibition of 12 to 59% was detected in all birds.

Response to carbofuran as indicated by labored breathing occurred as early as 2 to 5 min postdosage. All birds were sick and two males were dead within 15 to 30 min, but most appeared to have recovered and resumed feeding and other normal activities by 50 min. Brain ChE activity

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TABLE 2. Normal and inhibited cholinesterase activity[a] in Japanese quail blood plasma assayed fresh and after storage at 4 C for 1 to 2 days.

	Fresh			24 ± 1 hr			48 ± 1 hr		
Sex	Mean	SE	Extremes	Mean	SE	Extremes	Mean	SE	Extremes
				Control					
Male	1,518	64	1,217, 1,708	1,708[c]	83	1,392, 2,048	1,640	52	1,451, 1,825
Female	1,132[b]	43	936, 1,287	1,458[b,c]	47	1,287, 1,650	1,455[b,c]	53	1,287, 1,708
				Dicrotophos					
Male	73[b]	4	58, 82	76[b]	4	58, 94	73[b]	6	47, 94
Female	579	157	70, 1,416	526	149	70, 1,217	480	133	82, 1,205
				Carbofuran					
Male	153[b]	10	117, 211	155[b]	13	105, 199	154[b]	17	105, 246
Female	679	132	199, 1,288	579	138	187, 1,205	620	160	176, 1,381

[a] Cholinesterase activity (mU acetylthiocholine iodide hydrolyzed per min per ml of plasma at 25 C) was inhibited by administering a single oral dosage of 3 mg of chemical/kg of body weight. Blood was drawn at 1 hr ± 5 min posttreatment (n = 8 per data point). All dicrotophos and carbofuran means are significantly less than their respective controls ($P < 0.01$).
[b] Significantly less than cohorts of opposite sex ($P < 0.05$).
[c] Significantly different than fresh ($P < 0.05$).

of survivors was depressed ($P < 0.05$) by averages of 55 and 57% in females and males (Table 1). Overall, brain ChE inhibition ($P < 0.05$) varied from 31 to 79% in carbofuran-dosed quail.

Fresh plasma ChE activity averaged 34% higher ($P < 0.01$) for control males than for females (Table 2). This difference decreased to 17% ($P < 0.05$) during 24 hr of storage at 4 C as average ChE activities increased ($P < 0.01$) by 12 and 29% for males and females; ChE activity remained stable between 24 and 48 hr for both sexes. Storage at −25 C did not affect ChE activity in male plasma, but ChE activity in plasma of females increased by an average of 19% ($P < 0.05$) after 28 days and the sexes were statistically inseparable at this time (Table 3). The difference between sexes in average plasma ChE activity dur-

TABLE 3. Normal and inhibited cholinesterase activity[a] in Japanese quail blood plasma assayed fresh and after storage at −25 C for 1 to 28 days.

	Fresh			24 ± 1 hr			7 days	
Sex	Mean	SE	Extremes	Mean	SE	Extremes	Mean	SE
				Control				
Male	1,518	64	1,217, 1,708	1,539	82	1,088, 1,802	1,544	80
Female	1,132[b]	43	936, 1,287	1,252[b,c]	26	1,088, 1,322	1,303[b,c]	38
				Dicrotophos				
Male	73[b]	4	58, 82	73[b]	4	58, 94	80[b]	3
Female	579	157	70, 1,416	370	66	94, 632	308[c]	49
				Carbofuran				
Male	153[b]	10	117, 211	139[b]	7	105, 164	152[b]	18
Female	679	132	199, 1,288	524	100	152, 1,006	495[c]	84

[a] Cholinesterase activity (mU acetylthiocholine iodide hydrolyzed per min per ml of plasma at 25 C) was inhibited by administering a single oral dosage of 3 mg of chemical/kg of body weight. Blood was sampled at 1 hr ± 5 min posttreatment (n = 8 per data point). All dicrotophos and carbofuran means are significantly less than their respective controls ($P < 0.01$).
[b] Significantly less than cohorts of opposite sex ($P < 0.05$).
[c] Significantly different than fresh ($P < 0.05$).

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ing storage at −25 C for 0, 1, 7, and 28 days was 34 ($P < 0.01$), 22 ($P < 0.05$), 19 ($P < 0.05$) and 10% ($P > 0.05$), respectively, with males always highest.

Plasma ChE activity was almost completely inhibited (>95%) in the eight male quail dosed with 3 mg of dicrotophos per kg BW, but the same dosage resulted in significant inhibition (i.e., >21%, $P < 0.05$) in only six of eight females. Of these female respondents, only one was inhibited comparably to the average male response of 95% whereas the other five varied from 34 to 82%. In contrast to control ChE activity which increased during refrigeration, dicrotophos-inhibited plasma ChE activity was not affected by storage at 4 C for 2 days, but the net effect was an increase in the degree of apparent inhibition (Table 2). Fresh plasma ChE activity was inhibited by averages of 49 ($P < 0.05$) and 95% ($P < 0.01$) for females and males compared to 67 ($P < 0.01$) and 96% ($P < 0.01$) after refrigeration. Dicrotophos-inhibited ChE activity in plasma of male quail remained stable when stored at −25 C for 28 days just as for uninhibited plasma ChE (Table 3). Under the same conditions, activity of inhibited plasma ChE from breeding females decreased by an average of 56% ($P < 0.05$) and when combined with increased ChE of control female plasma, the result was an overall increase from 49% (fresh) to 82% during 28 days at −25 C.

ChE activity was inhibited to about the same degree in plasma of quail dosed with 3 mg of carbofuran per kg BW as was indicated for dicrotophos (Table 2). Likewise, neither male nor female ChE activity was affected by refrigeration at 4 C for 2 days or for males by freezing at −25 C for 28 days, but again female plasma ChE activity continued to decrease while frozen at −25 C (cf. Tables 2 and 3). The net effect of the divergent responses of control and carbofuran-inhibited plasma ChE activity was to increase the degree of inhibition ($P < 0.01$) for females from an initial average of 40% to 57% after 2 days at 4 C and 67% after 28 days at −25 C.

TABLE 3. Continued.

7 days	28 days		
Extremes	Mean	SE	Extremes
Control			
1,310, 1,954	1,476	75	1,275, 1,872
1,147, 1,427	1,348[c]	32	1,252, 1,521
Dicrotophos			
70, 94	72[b]	5	58, 94
94, 515	256[c]	47	47, 456
Carbofuran			
82, 257	158[b]	17	105, 211
187, 866	442[c]	70	211, 796

DISCUSSION

The potentially lethal oral dosage of 3 mg of carbofuran or dicrotophos per kg BW given to reproductively active Japanese quail resulted in significant brain and blood plasma ChE inhibition in all subjects and killed 10 to 15% of the birds. Patterns of response and recovery were very different for the two chemicals. All quail administered carbofuran were affected almost immediately and either died or appeared to have recovered within about 30 min. Both sexes seemed to have recovered within 1 hr postdosage although brain ChE inhibition of 60% was detected in one half of the birds. Rapid recovery of quail from acute carbofuran poisoning had been previously reported, but corresponding brain ChE activity was not evaluated (Hill and Camardese, 1984). Spontaneous reactivation of carbamylated brain ChE was therefore presumed to be primarily responsible for the rate of recovery. The present study demonstrated that reversal of overt signs of acute carbofuran toxicity was possible without concomitant recovery of whole brain ChE activity. This may support the hypothesis that an alternative

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mechanism such as neuromuscular blocking rather than central nervous system intervention may be responsible for rapid deaths often attributed to carbamate exposure (Westlake et al., 1981b). It is also possible that differential rates of ChE inhibition and recovery in critical regions of the brain are masked by use of whole brain ChE activity for diagnostic purposes, but differential rates of response among the regions and importance to toxicity are only conjectural.

In contrast with carbofuran, time to onset of intoxication from dicrotophos was variable, but once noted it seemed to intensify in all birds until most were ataxic by 30 to 45 min postdosage. Thereafter, dicrotophos-dosed birds either died or appeared to stabilize and, although moderate ataxia persisted in most birds at the time of euthanasia, none appeared critically intoxicated but neither did any recover to the extent apparent for carbofuran-dosed quail. Although the two chemicals were about equally toxic in terms of lethality at the dosage tested, the effects of dicrotophos were more protracted and consuming over time, yet brain ChE activity was depressed by an average of about 35% ($P < 0.05$) further for "recovered" carbofuran-dosed birds than their obviously sick dicrotophos-dosed counterparts (Table 1). This relationship was probably coincidental because the endpoint was timed rather than based on a biological response and carbamylation of ChE proceeds much faster than phosphorylation (O'Brien, 1976). At the appointed time of euthanasia, brain ChE was likely descending in dicrotophos-dosed birds due to incomplete phosphorylation of enzyme while ChE may actually have been ascending in carbofuran-dosed birds due to spontaneous reactivation of carbamylated enzyme.

Sex effects were not detected in brain ChE activity of normal or dosed Japanese quail or in their clinical response to either chemical although 90% of the female subjects were heavier and all appeared fatter than any male tested. However, blood plasma ChE activity consistently differed ($P < 0.05$) between the sexes of these reproductively active quail and the relationship was opposite for uninhibited and inhibited ChE (Table 2). In a study of northern bobwhites (*Colinus virginianus*) at different seasons of the year, sex differences in plasma ChE activity only occurred while birds were in reproductive condition and at that time ChE activity was highest for males (Hill and Murray, 1987). In laboratory rats, reproductively active females may have plasma ChE activity as much as three to five times the level of males (Illsley and Lamartiniere, 1981). Sex steroids were shown responsible for suppression of plasma ChE in male rats (androgen) and stimulation of ChE in females (estrogen), but no comparable studies were found to explain why this relationship is opposite for birds.

It is not clear why female plasma ChE activity was not usually as responsive to either dicrotophos or carbofuran as was male ChE when all birds were dosed the same in relation to body weight and such dosage resulted in equivalent inhibition of brain ChE in the two sexes, or why plasma ChE inhibition was so much more variable for females than males. However, because activity of inhibited female plasma ChE gradually decreased over time while frozen and male plasma ChE activity remained constant, some constituent in breeding female blood may have formed a weak bond with certain unidentified plasma nonspecific esterases and initially interfered with antiChE-ChE complexing. Slow freezing and storage at −25 C may have dissociated such a complex as indicated by increased control ChE activity and thereby freed ChE for more permanent binding with antiChE (Table 3). Refrigeration at 4 C resulted in significant elevation in control ChE activity for both sexes while inhibited ChE activity was not significantly altered for either sex.

Whether these sex differences in plasma ChE activity influenced tolerance of antiChE exposure could not be deter-

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mined by the present experiment, but because brain ChE was inhibited equally for the sexes (Table 1) it would seem that toxicity mediated through the central nervous system should not be affected. If antiChE toxicity is mediated through alternative mechanisms such as neuromuscular intervention as suggested for carbamate exposure (Westlake et al., 1981b), then some protection may be possible. Any degree of protection would be expected to be quite variable because the level of plasma esterase activity is highly variable among bird species (Westlake et al., 1983).

In conclusion, blood plasma ChE activity varied between the sexes of reproductively active birds and storage of plasma at −25 C for durations to 28 days had different effects on uninhibited (control) and inhibited ChE acitivity in female plasma. At the same time, neither uninhibited, carbamylated, nor phosphorylated male plasma ChE activity was affected by storage at −25 C. Control female plasma ChE activity increased significantly (≈20%) while carbamylated and phosphorylated ChE decreased by averages of about 35 and 55%. These confounding patterns of ChE activity indicate extreme care should be exercised in use of plasma for evaluation of antiChE exposure in wild birds, but none of the potential sources of error detected in this study would interfere with detection of exposure.

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