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FURTHER STUDIES OF BRAIN CHOLINESTERASE: CHOLINERGIC RECEPTOR RATIOS IN THE DIAGNOSIS OF ACUTE LETHAL POISONING OF BIRDS BY ANTICHOLINESTERASE PESTICIDES

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ABSTRACT: Cholinesterase (ChE) and the muscarinic (mAChR) and nicotinic (nAChR) cholinergic receptors were measured in the brains of 86 birds of 20 different species collected in Saskatchewan, Canada during 1991 and 1992. There was a strong correlation between ChE and mAChR, and the ratio of ChE : mAChR was 38% less variable than was ChE alone. In a hypothetical test for minimal acute lethal poisoning of all birds based on a reduction by 50% of the normal ChE measured in each, the use of a single diagnostic threshold value of the ChE : mAChR ratio had a diagnostic sensitivity of 98% and a specificity of 99%, thus making theoretically possible the diagnosis of acute lethal poisoning by anticholinesterase chemicals without the use of reference control values for ChE. Both ChE and mAChR were highly stable during 12 days of postmortem decomposition under several different daily temperature regimes. Substantial changes in these molecules occurred only under conditions of constant high temperature (36 C). Acute lethal poisoning by the organophosphate chlorpyrifos did not affect the density of mAChR. Postmortem decomposition did not appear to be an important confounding factor in the diagnostic interpretation of either ChE or the ChE : mAChR ratio except under hot climatic conditions.

Key words: Pesticide, poisoning, diagnosis, cholinesterase, muscarinic cholinergic receptor, bird.

INTRODUCTION

Because of the high cost of chemical analysis for anticholinesterase pesticides and their metabolites in animal tissues and stomach content, the diagnosis of acute lethal poisoning by these compounds often is based on the relatively inexpensive measurement of the activity of brain cholinesterase (ChE). Depression of ChE activity to ≤50% of normal is the widely-accepted standard for diagnosis of lethal intoxication established by Ludke et al. (1975). However, diagnostic interpretation of ChE data requires knowledge of the normal ChE value for each species, since there can be three-fold differences among some avian species (Hill, 1988; Blakley and Skelley, 1988), and it may be confounded as well by changes in ChE levels due to post-mortem decomposition. The suggestion that interpretation of ChE should always be based on comparison with concurrent measurements in control animals of the same species, preferably of the same age and sex, and exposed to the same post mortem conditions as the diagnostic specimens themselves, is sound but impractical (Hill and Fleming, 1982). To overcome the potential problem of postmortem decomposition in diagnostic specimens, Prijono and Leighton (1991) introduced the use of the ratio of ChE and the density of muscarinic acetylcholine receptors (mAChR), molecules in the biochemical cycle of cholinergic transmission the density of which is unaffected by acutely lethal poisoning with anticholinergic compounds. The current study was undertaken for two purposes: 1) to test the hypothesis that, in contrast to ChE alone, the ChE : mAChR ratio is sufficiently similar among bird species to obviate the need for control specimens in the diagnosis of acute, lethal anticholinesterase poisoning; and 2) to further test the stability of the ChE : mAChR ratio under a variety of conditions of post mortem decomposition in normal and poisoned birds.

MATERIALS AND METHODS

Two experiments were conducted during 1991 and 1992. In the first, brains were ob-

TABLE 1. Twenty avian species used for the comparison of interspecies variability of ChE activities and ChE : mAChR ratios.

Common name	Scientific name	Number tested
House sparrow	*Passer domesticus*	13
Yellowheaded blackbird	*Xanthocephalus xanthocephalus*	10
American coot	*Fulica americana*	10
Mallard	*Anas platyrhynchos*	10
Japanese quail	*Coturnix coturnix japonicus*	10
Bald eagle	*Haliaeetus leucocephalus*	8
Least sandpiper	*Calidris minutilla*	5
Great blue heron	*Ardea herodias*	3
Tundra swan	*Cygnus columbianus*	2
Canada goose	*Branta canadensis*	2
Lesser yellowlegs	*Tringa flavipes*	2
Semipalmated sandpiper	*Calidris pusilla*	2
Cedar waxwing	*Bombycilla cedrorum*	2
Green-winged teal	*Anas crecca*	1
Canvasback	*Aythya valisineria*	1
Northern harrier	*Circus cyaneus*	1
Lesser scaup	*Aythya affinis*	1
Bufflehead	*Bucephala albeola*	1
Long-eared owl	*Asio otus*	1
Red-tailed hawk	*Buteo jamaicensis*	1

tained from 86 birds of 20 different species for measurement of the activity of ChE and the density of mAChR and of the nicotinic cholinergic receptor (nAChR). Specimens of yellowheaded blackbird (*Xanthocephalus xanthocephalus*), American coot (*Fulica americana*), house sparrow (*Passer domesticus*) and mallard (*Anas platyrhynchos*) were trapped or shot under permit. Japanese quail (*Coturnix coturnix japonicus*) were obtained from a colony maintained by the Peregrine Falcon Project, Western College of Veterinary Medicine (WCVM), University of Saskatchewan, Saskatoon, Canada. Specimens of 15 other species (Table 1) were obtained from animals submitted to the Diagnostic Laboratory of the Department of Veterinary Pathology, WCVM, for which a cause of death not associated with exposure to anticholinesterase pesticides was clearly evident. Brains were frozen and stored at −80 C until analyzed.

The second experiment examined the same molecules during decomposition of the brain. Heads of freshly-killed Japanese quail were obtained from the Peregrine Falcon Project, WCVM. Groups of 10 heads were placed in plastic bags and subjected to one of three temperature regimes for periods of 0, 4, 8, 12 or 16 days. The temperature regimes were alternation between 3 C and 21 C at 12-hr intervals, alternation between −20 C and 21 C at 12-hr intervals, or a constant 36 C. Humidity was neither measured nor controlled. At the end of the predetermined period of time, each bag was sealed and the heads were frozen at −80 C until analyzed.

For study of decomposition in brains from poisoned birds, 60 Japanese quail were obtained from the Peregrine Falcon Project, WCVM, and maintained in cages together for 4 days. Fifty then were given 32 mg/kg of the organophosphate pesticide chlorpyrifos (Dow-Elanco Canada Inc., Saskatoon), twice the published 50% lethal oral dose (Smith, 1987), diluted in corn oil and administered directly into the crop. Birds that survived 90 min after exposure were given an additional oral dose of the same amount. Three birds that still survived after one additional hour were killed with CO_2 and were eliminated from the experiment. The 10 remaining birds received an oral dose of corn oil only and were killed with CO_2 2 hr later. After death, the heads of the control birds and of 10 poisoned birds were placed in plastic bags and frozen at −80 C. The remaining heads from poisoned quail were placed in plastic bags in groups of nine or 10 and exposed to a temperature regime alternating at 12-hr intervals between 3 C and 25 C for 4,8,12, and 16 days. An additional 40 heads from freshly-killed quail were obtained from the Peregrine Falcon Project, placed in plastic bags in groups of 10, and exposed to the same temperature regime at the same time as the heads from poisoned quail. At the end of each decomposition period, the bags were sealed and frozen at −80 C until analyzed.

For all analyses, a 1:10 weight : volume homogenate of brain tissue was prepared from half of each brain, obtained by cutting partially-thawed heads in the mid-sagittal plane and removing the still-solid brain tissue from one half. All chemicals were obtained from Sigma Chemical Company (St. Louis, Missouri, USA) unless otherwise indicated. Brains were homogenized in 0.05 M sodium/potassium phosphate buffer, pH 7.7 (Na/K buffer) using a Brinkmann Polytron PT 3000 homogenizer (Brinkmann Instruments, Rexdale, Ontario, Canada) at 30,000 rpm for 30 sec. Homogenates then were refrozen at −80 C in 0.5 ml aliquots. Homogenates in tris buffer were found advantageous in assays for the nAChR. These were prepared by centrifugation of the Na/K-buffered homogenates at 6,000 × *G* for 20 min, removing the supernatant and replac-

ing it with an equal volume of 0.05 M tris buffer, pH 7.4, in which the pellet was re-suspended by means of the Polytron homogenizer. The centrifugation and resuspension procedure was repeated twice.

Measurements of ChE, mAChR and nAChR each were done in triplicate for each specimen and the average of the three measurements was used as the specimen value. Brain ChE activity was measured by the method of Ellman et al. (1961) as modified by Hill and Fleming (1982). The reaction was carried out at 22 ± 1 C and all reactants were warmed to this temperature prior to initiating the reaction. Change in absorbance at 405 nm with a 2 nm band width was measured every 30 sec for 2.5 min. Calculations were based on a molar extinction coefficient for 5-thio(2-nitrobenzoate) of 1.36×10^4 M^{-1} cm^{-1} (Fairbrother et al., 1991).

The density of mAChR was determined by a modification of the method of Yamamura and Snyder (1974). Modifications were undertaken to achieve receptor saturation with a minimum of radioactive ligand. A high (1.45 mM) concentration of oxotremorine was used to ensure near-complete displacement of the ligand for evaluation of non-specific binding. Less than 15% non-specific binding was achieved without requirement for a centrifugation step. For the assay, all reagent solutions were prepared immediately prior to the procedure. Each tube was filled with either 430 µl 0.05M Na/K buffer or 380 µl Na/K buffer and 50 µl oxotremorine solution (1.45 mM in Na/K buffer). Twenty µl of brain homogenate was added and the tubes were mixed and left at 22 ± 1 C for 10 min. Fifty µl of [^{3}H]QNB solution (10 nM [(R)-1-Quinuclidinyl[phenyl-4(n)^{3}H] Benzilate (Amersham Canada Ltd., Oakville, Ontario, Canada.) in ethanol with specific activity of 41.6 Ci/mmol then was added. The tubes were mixed and incubated for 1 hr at 22 ± 1 C. The reaction was stopped by addition of 4 ml ice cold Na/K buffer and rapid filtration onto Whatman GF/B discs, 2.4 cm in diameter (Fisher Scientific, Edmonton, Alberta, Canada). These were quickly rinsed with four volumes of 4 ml of cold buffer and then placed in scintillation counting vials to which 10 ml of CytoScint ES liquid scintillation solution (I.C.N. Biomedicals, Mississauga, Ontario, Canada) was added. Vials were held at 20 C for at least 12 hr and then were shaken vigorously immediately prior to counting for 10 min in a Beckmann LS 8000 scintillation counter (Beckmann Instruments, Mississauga, Ontario). Further procedural details are given in Burn (1993).

The density of nAChR was measured by the method of Schwartz et al. (1982), based on the binding of [^{3}H]-acetylcholine (Amersham Canada Ltd). Reagent solutions were prepared within a few hours of their use. To minimize non-specific binding, reaction tubes were kept partially immersed in ice water and the glass fiber filters were pre-wetted with 400 µl of polyethylenimine solution (0.05% in tris buffer). Scintillation counting was done as in the mAChR procedure.

The relative diagnostic utility of measurements of ChE and of the ChE : mAChR ratio were estimated by comparing the actual values of these molecules in the brains of 86 individual birds of 20 species with values that would result from a 50% reduction of ChE activity in each bird. This calculation was intended to represent the minimum change in ChE associated with acute lethal poisoning by anti-cholinesterase compounds.

The mean ChE : mAChR and ChE : nAChR ratios for each species were calculated as the average of ratios for each individual bird except for the ChE : nAChR ratio for quail. In this case, a mean nAChR was determined by nine separate assays of pooled brain homogenate from nine quail and this value was used in the calculation of the ratio. Descriptive statistics, one-way analysis of variance to compare two means, and correlation/regression analyses were done using the Minitab statistical program (Release 7.1, Minitab Inc., State College, Pennsylvania, USA). Three or more means were compared by one-way analysis of variance and the Tukey-HSD multiple range procedure of the SPSS statistical computing program (Release 4.1, SPSS Inc., Chicago, Illinois, USA).

RESULTS

The coefficient of variation of the ChE : mAChR ratio for species with sample sizes over eight was approximately half that of ChE alone or of the ratio of ChE : nAChR (0.17 versus 0.30 or 0.32) (Table 2). The correlation coefficient (r) for ChE and mAChR in the brains of 86 individual birds of 20 different species was 0.83. Regression analysis gave $R^2 = 0.68$ and analysis of variance of the regression indicated highly significant ($P < 0.001$) interdependence of the activity of ChE and the density of mAChR (Fig 1).

The degree of overlap between normal values and those with ChE reduced by 50% was considerably greater for ChE alone than for the ChE : mAChR ratio (Fig 2); 38% of the normal ChE values fell within the range for minimally poisoned

TABLE 2. Mean cholinesterase activity (ChE), density of muscarinic (mAChR) and nicotinic (nAChR) receptors and ChE : receptor ratios for six avian species of which eight or more individuals were tested. Means of species means are given at the bottom of the table.

Species	Number tested	ChE[a]	mAChR[b]	nAChR[b]	ChE : mAChR ratio	ChE : nAChR ratio
Yellow-headed blackbird	10	21.7[c]	41.53	1.90	0.520	11.61
		(1.71)	(1.49)	(0.284)	(0.046)	(2.04)
American coot	10	21.4	40.86	2.96	0.527	7.42
		(3.62)	(3.55)	(0.651)	(0.093)	(1.48)
House sparrow	13	24.5	45.10	2.63	0.546	9.39
		(2.62)	(4.02)	(0.313)	(0.063)	(1.19)
Mallard	10	12.1	31.25	ND[d]	0.386	ND
		(1.40)	(2.89)		(0.037)	
Bald eagle	8	15.2	27.44	ND	0.559	ND
		(1.86)	(3.80)		(0.071)	
Japanese quail	10	12.0	31.54	2.30	0.383	5.24[e]
		(0.71)	(2.95)	(0.184)	(0.025)	
Mean	6	17.8	36.29	2.45	0.487	8.42
Standard deviation		5.40	7.10	0.454	0.081	2.72
Coefficient of variation		0.303	0.196	0.185	0.165	0.324

[a] μmoles of acetylthiocholine hydrolyzed per minute per gram wet tissue.
[b] Moles per gram wet tissue.
[c] Mean (standard deviation).
[d] ND, no data.
[e] This ratio was calculated using mean ChE and nAChR values, hence, no SD is given.

birds while 52% of the values for minimally-poisoned birds fell within the range of normal ChE values. The corresponding percentage overlaps for the ChE : mAChR ratios were 6% and 3% respectively.

Both ChE and mAChR molecules were remarkably resistant to degradation during postmortem decomposition. The maximum loss of ChE activity after 16 days of decomposition was 9% under all conditions except constant high temperature (Table 3). The stability of mAChR was similar during 12 days of decomposition (Table 4). The ratios of ChE : mAChR changed minimally during 12 days of decomposition except at a constant temperature of 36C (Table 5).

In quail poisoned lethally with chlorpyrifos, ChE was reduced by 91% relative to controls at the time of death (Table 6). Cholinesterase activity recovered to 53% of the control value after 16 days of postmortem decomposition at 3 C to 21 C. The mean mAChR of poisoned birds immediately after death (day 0) was similar to control groups from this and other (Table 4) experiments, and had stability similar to that of controls during postmortem decomposition. Means of both ChE and the ChE : mAChR means were significantly ($P < 0.05$) and substantially different from control means during 12 days of decomposition. On day 16, mean ChE remained significantly ($P < 0.05$) below the control mean but was no longer reduced by the usual diagnostic threshold of 50% below the control mean.

DISCUSSION

Diagnosis of acute lethal poisoning due to anticholinesterase chemicals by measurement of ChE requires some reasonable estimate of the normal level of ChE activity for the animal in question. This may be done by concurrent measurement of ChE in suitable control specimens or by reference to published data regarding normal ChE levels for various species. Serious problems arise in both cases. Collection and killing of additional animals to serve

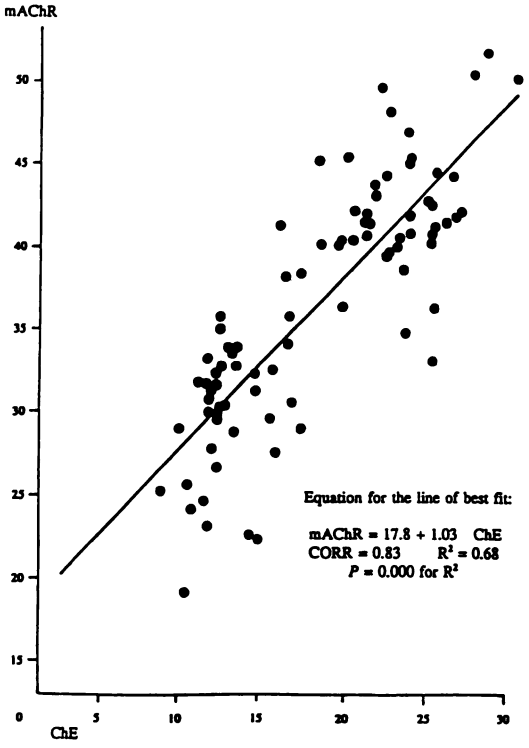

FIGURE 1. Linear correlation of density of muscarinic cholinergic receptors (mAChR) with activity of cholinesterase (ChE) in the brains of 86 individual birds of 20 different species (see Table 1). CORR = correlation coefficient.

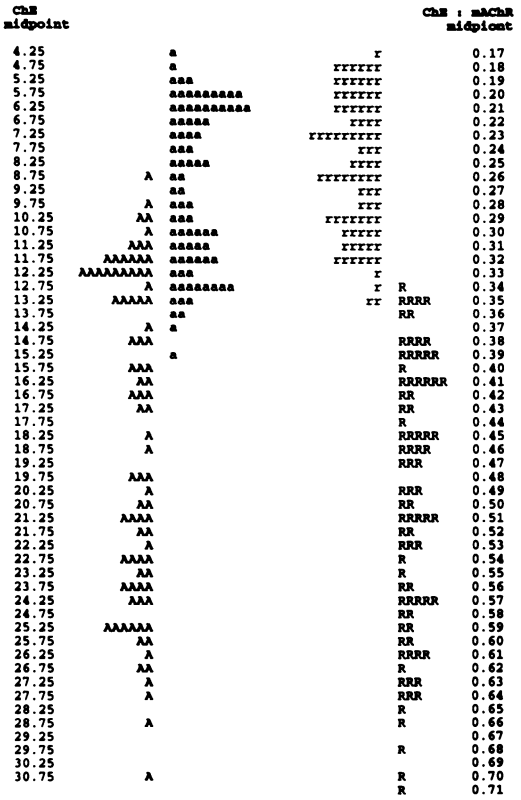

FIGURE 2. Theoretical comparison of the diagnostic utility of measuring brain ChE alone (A) and of the ChE : mAChR ratio (R). Normal values (upper case) for 86 individual birds are compared with the same values when the normal ChE is reduced by 50% (lower case): the minimum criterion for acute lethal poisoning by anticholinesterase chemicals. The degree of overlap indicates the probability of error in diagnostic interpretation when the normal value of ChE for the species in question is not known.

as controls may be neither feasible nor desirable. There is considerable inconsistency in the published reference values for ChE, with nearly two-fold differences in the maxima and minima reported by different authors for the same species (Zinkl et al., 1977; Hill and Fleming, 1982; Westlake et al., 1983; Hill, 1988). These differences may be due to differences in the details of the application of the Ellman method of enzyme measurement. Many of the published values are based on measurements of only a few individuals of each species. Furthermore, these values are available for less than 150 species of North American and European birds while in North America alone their are 650 breeding species of birds and an additional 150 or so species that are occasional visitors (National Geographic Society, 1987).

The ChE : mAChR ratio offers another approach to the diagnostic interpretation of ChE in brain. The highly significant correlation between ChE and mAChR (Fig. 1) among species with very different ChE activities is evidence for a fixed relationship between the two molecules as measured in this study, possibly regulated to maintain an optimal balance for impulse transmission across muscarinic cholinergic synapses. Among all species tested, the ChE : mAChR ratio was 38% less variable than was ChE alone. This was not the case for the ratio with nAChR, and, for this rea-

TABLE 3. Brain cholinesterase activity (ChE) of Japanese quail following *post-mortem* decomposition under four different environmental conditions.

	Days after death				
Temperature	0	4	8	12	16
3 C to 21 C	12.02[a]	11.83	11.33	11.69	11.94
	(0.71)	(0.98)	(0.73)	(0.73)	(0.70)
3 C to 25 C	13.11	12.22[b]	11.84[b]	11.84[b]	12.02[b]
	(0.50)	(0.61)	(0.84)	(0.65)	(1.67)
−20 C to 21 C	12.02	10.06[b]	11.05	11.88	12.11
	(0.71)	(0.52)	(0.56)	(1.44)	(1.08)
36 C	12.02	9.47	7.21[b]	ND[c]	ND
	(0.71)	(4.86)	(5.27)		

[a] Mean (standard deviation) from measurement of 10 brains; units are μmoles of acetylthiocholine hydrolysed per min per g wet tissue.
[b] Significantly different than day 0 at $P \leq 0.05$, analysis of variance with Tukey-HSD procedure.
[c] ND, no data.

son, nAChR was not explored further. Because of the high correlation between the activity of ChE and the density of mAChR in avian brains, the ChE : mAChR ratio can be interpreted without reference to control specimens or previous knowledge of the normal activity of ChE for the species of bird in question. Based on Fig. 2, we selected 0.345, the center of overlap, as a theoretical threshold value for the ChE : mAChR ratio, with any value below this taken as evidence of acute lethal poisoning by anti-cholinesterase chemicals. For the data in Fig. 2, this diagnostic threshold had a sensitivity (true positive rate) of 98% and a specificity (true negative rate) of 99% (Sackett et al., 1985). These are entirely acceptable for diagnostic procedures. By contrast, similar interpretation of ChE alone, using 12.50 as the threshold value, yields a sensitivity of 78% and a specificity of 83%. The measurement of both ChE and mAChR are relatively easy and quite inexpensive. However, both require basic laboratory equipment, rigorous attention to procedural details and regular use of control specimens for standardization in order to produce data that are comparable from one assay to another. The amount of [^{3}H]QNB required for mAChR measurements is very small.

Postmortem decomposition was found to be only a minor confounding factor in

TABLE 4. Density of muscarinic cholinergic receptor (mAChR) in brains of Japanese quail following *post-mortem* decomposition under four different environmental conditions.

	Days after death				
Temperature	0	4	8	12	16
3 C to 21 C	31.54[a]	32.27	28.38	27.99	24.00[b]
	(2.95)	(2.06)	(1.90)	(3.29)	(8.64)
3 C to 25 C	38.26	32.15	24.65	27.18	20.82
	(1.66)	(1.82)	(4.32)	(6.38)	(12.93)
−20 C to 21 C	31.54	26.93	28.55	26.76	21.07[b]
	(2.95)	(2.64)	(4.93)	(4.17)	(4.89)
36 C	31.54	5.43[b]	1.01[b]	ND[c]	ND
	(2.95)	(3.75)	(1.10)		

[a] Mean (standard deviation) from measurement of 10 brains; units are moles per g wet tissue.
[b] Significantly different than day 0 at $P \leq 0.05$, analysis of variance with Tukey-HSD procedure.
[c] ND, no data.

TABLE 5. Mean ratio of cholinesterase activity to muscarinic cholinergic receptor density (ChE : mAChR) following *post-mortem* decomposition under four different environmental conditions.

	Days after death				
Temperature	0	4	8	12	16
3 C to 21 C	0.383[a]	0.367	0.400	0.423	0.85
	(0.025)	(0.033)	(0.030)	(0.054)	(1.19)
3 C to 25 C	0.344	0.380	0.493	0.464	3.11
	(0.024)	(0.016)	(0.083)	(0.137)	(6.74)
−20 C to 21 C	0.383	0.377	0.400	0.450	0.64[b]
	(0.025)	(0.039)	(0.086)	(0.062)	(0.32)
36 C	0.383	2.46	25.2	ND[c]	ND
	(0.025)	(1.71)	(49.1)		

[a] Mean (standard deviation) from measurement of 10 brains.
[b] Significantly different than day 0 at $P \leq 0.05$, analysis of variance with Tukey-HSD procedure.
[c] ND, No data.

the interpretation of ChE and mAChR except under hot climatic conditions. Both molecules were highly resistant to degradation for up to 12 days after death except under conditions of constant high temperature. It probably is rare that birds that die in the field will be discovered after 12 days (Wobeser et al., 1982; Wobeser and Wobeser, 1992). Thus, postmortem decomposition largely can be discounted as a confounding factor in diagnostic interpretation in temperate and cold climates.

The mechanism by which ChE activity increased during postmortem decomposition in quail poisoned with chlorpyrifos was not explored in this study. Both reactivation of ChE and aging, which resists reactivation, can occur after intoxication with organophosphates (Wilson et al., 1992; Johnson et al., 1993). Elevations of ChE above day 0 control levels also were noted in some individual brains after decomposition at 36 C for 4 and 8 days. This might be accounted for by the production of cholinesterase by decomposing bacteria (Garber and Nachshon, 1980) or by activation of a pool of catalytically-inactive ChE (Chatel et al., 1993).

TABLE 6. Cholinesterase activity (ChE), density of muscarinic cholinergic receptor (mAChR) and ChE : mAChR ratios in brains from Japanese quail untreated or poisoned with chlorpyrifos and undergoing *post-mortem* decomposition at 3 to 25 C.

	ChE		mAChR		ChE : mAChR	
Days	Control	Treated	Control	Treated	Control	Treated
0	13.11[a]	1.25[d]	38.26	34.18[d]	0.344	0.037[d]
	(0.50)[b]	(0.25)	(1.66)	(3.89)	(0.024)	(0.010)
4	12.22	4.18[c,d]	32.15	34.79[d]	0.380	0.121[c,d]
	(0.61)	(0.30)	(1.83)	(2.88)	(0.016)	(0.014)
8	11.84	5.71[c,d]	24.65	29.59[c,d]	0.493	0.198[c,d]
	(0.49)	(0.47)	(4.32)	(3.87)	(0.083)	(0.040)
12	11.84	6.04[c,d]	27.18	28.84	0.464	0.210[d]
	(0.65)	(0.34)	(6.38)	(2.01)	(0.137)	(0.012)
16	12.02	6.97[c,d]	20.82	18.20[c]	3.11	0.81[c]
	(1.07)	(0.67)	(12.93)	(10.80)	(6.74)	(0.99)

[a] Mean ($n = 10$ unless otherwise noted).
[b] () Standard deviation.
[c] $n = 9$.
[d] Significantly ($P < 0.05$) different than untreated controls, by analysis of variance.

Acute lethal poisoning with chlorpyrifos did not appear to affect mAChR density. At various times during decomposition, the mAChR from poisoned birds was both higher and lower than the respective controls. Thus, while chronic ChE inhibition or prolonged stimulation by muscarinic agonists can cause reduction in the density of avian and mammalian mAChR (Sivam et al., 1983; Hosey, 1992; Yang et al., 1993), acute exposure in this and previous experiments did not produce this effect (Prijono and Leighton, 1991).

We do not propose that diagnostic laboratories accept the ChE : mAChR ratio threshold value of 0.345, used here by way of illustration, as a diagnostic criterion for acute lethal poisoning by anticholinesterase chemicals. A larger data set and more rigorous analysis will be required to establish diagnostic thresholds. Furthermore, rigid procedural standardization among laboratories will be essential if threshold values for this ratio of two different biochemical measurements are established for general use.

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