AGE-DEPENDENT CHANGES IN PLASMA AND BRAIN CHOLINESTERASE ACTIVITIES OF EASTERN BLUEBIRDS AND EUROPEAN STARLINGS

Authors: Gard, Nicholas W., and Hooper, Michael J.

Source: Journal of Wildlife Diseases, 29(1) : 1-7

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-29.1.1

Journal of Wildlife Diseases, 29(1), 1993, pp. 1–7

AGE-DEPENDENT CHANGES IN PLASMA AND BRAIN CHOLINESTERASE ACTIVITIES OF EASTERN BLUEBIRDS AND EUROPEAN STARLINGS

Nicholas W. Gard and Michael J. Hooper

The Institute of Wildlife and Environmental Toxicology and Department of Environmental Toxicology, Clemson University, P.O. Box 709, Pendleton, South Carolina 29670, USA

ABSTRACT: Age-dependent changes in plasma and brain cholinesterase (ChE) activity were characterized in two altricial passerine species: eastern bluebirds (*Sialia sialis*) and European starlings (*Sturnus vulgaris*). Plasma acetylcholinesterase (AChE) activity declined rapidly immediately after hatching, while plasma butyrylcholinesterase (BChE) activity increased throughout the nestling period. These patterns continued after birds fledged, since the BChE : AChE ratio was higher in adult birds than fledglings. This is the first confirmation of age-dependent changes in plasma ChE activity in altricial species. Total plasma ChE activity increased with age in both species, which is the reverse of results previously reported for several precocial species. Brain ChE activity increased with age in both species, and did not reach asymptotic levels before young fledged. This corresponded with patterns previously documented in European starlings and three other altricial species. We propose that age and degree of precocity in young birds must be considered when examining sensitivity or evaluating field exposure of birds to ChE-inhibiting compounds.

Key words: Brain cholinesterase, plasma cholinesterase, eastern bluebird, *Sialia sialis*, European starling, *Sturnus vulgaris*, age-dependent changes.

INTRODUCTION

Development patterns of brain cholinesterase (ChE) activity of young altricial and precocial avian species are distinctly different. Age-dependent increases in brain ChE activity have been documented in altricial European starlings (*Sturnus vulgaris*) (Grue et al., 1981; Grue and Hunter, 1984; Robinson et al., 1988), and three species of altricial colonial waterbirds: black-crowned night-herons (*Nycticorax nycticorax*), great egrets (*Casmerodius albus*), and snowy egrets (*Egretta thula*) (Custer and Ohlendorf, 1989), suggesting that this may be a trait common to most altricial species. In precocial and semi-precocial species, ChE activity reaches adult levels during embryonic development; thus, hatchlings display brain ChE activity levels similar to adults (Ludke et al., 1975; White et al., 1979; Hoffman and Eastin, 1981; Farage-Elawar, 1991). Diagnosis of avian exposure to ChE-inhibiting organophosphorus (OP) and carbamate pesticides typically involves evaluation of brain ChE enzymes for depression in activity below control levels. Therefore, accurate diagnosis of ChE inhibition requires that enzyme activity in birds suspected of exposure be compared with controls of equal age.

Age-dependent differences in plasma ChE activity have been noted in young of several precocial species. Ludke et al. (1975) observed significantly higher total plasma ChE levels in 2-wk-old Japanese quail (*Coturnix japonica*) chicks than in 4- or 8-wk-old birds. Comparable findings have been reported in chickens (*Gallus domesticus*) (Lyles et al., 1980; Smucker and Wilson, 1990; Farage-Elawar, 1991) and mallards (*Anas platyrhynchos*) (Fairbrother et al., 1990; Bennett and Bennett, 1991). In both chickens and mallards, total plasma ChE activity decreased with age, largely due to a rapid decrease in activity of the acetylcholinesterase (AChE) component immediately after hatch. Plasma butyrylcholinesterase (BChE) activity was more variable, but also tended to decline as birds grew (Lyles et al., 1980; Bennett and Bennett, 1991). Age-dependent patterns of plasma ChE activity have not been evaluated for altricial species. However, since large differences exist in age-dependent brain ChE activity between altricial

and precocial species, differences might also be anticipated for plasma ChE activity.

Since plasma esterases are inhibited faster and more extensively by OP's than is brain ChE (Ludke et al., 1975; Westlake et al., 1981), plasma esterases provide a more sensitive indicator of OP toxicity. Additionally, plasma ChE analysis provides a means of detecting sub-lethal OP exposure, while also allowing repeated evaluations from a single individual (Fairbrother et al., 1989; Hooper et al., 1990). However, the effectiveness of plasma ChE analysis as a diagnostic tool requires the characterization of age-dependent changes in enzyme activities.

The objective of this study was to determine if changes in plasma AChE and BChE activity in nestlings of two altricial species, European starlings and eastern bluebirds (*Sialia sialis*), are age-dependent. Brain ChE activity also was measured in nestlings to compare age-dependent changes between species.

MATERIALS AND METHODS

Nestling European starlings were collected from nest boxes on two 16 ha field sites in Whatcom County (48°52'N, 122°36'W), Washington (USA) in 1989. Nestling eastern bluebirds came from nest boxes on pastures in Pickens (34°35'N, 82°47'W) and Anderson (34°40'N, 82°42'W) Counties, South Carolina (USA) in 1990. At each day of age from hatching to fledging (at approximately 21 days), 10 starlings were bled and then euthanized by carbon dioxide (CO_2) asphyxiation for subsequent brain ChE analysis. Bluebirds were bled at 2-day intervals from hatching to fledging (at approximately 16 days). Five to 15 bluebirds were bled at each age. No more than one bluebird or starling per nest was used at each age, and no bird was bled more than once. To obtain serial data on plasma ChE profiles of individual bluebirds and to assess the effects of multiple bleeding, an additional four young from four nests were sampled sequentially at 4-day intervals from 3 to 15 days of age. Birds younger than 3 days were not sampled, since the volume of blood that could be drawn non-lethally was insufficient for analytical purposes. To characterize brain ChE activity, five nestling bluebirds were euthanized by CO_2 asphyxiation at 4-day intervals from day 0 to 16. Blood samples from these birds were used in plasma ChE analyses. Two other nestlings euthanized by CO_2 asphyxiation at 3 and 14 days of age also were analyzed for brain ChE activity. All carcasses were stored whole at −20 C prior to analysis. Plasma samples and brains of 12 adult starlings (sex not determined) and eight adult bluebirds (four male, four female) were used to contrast nestling and adult ChE activity.

For each species, blood samples were always taken between 09:00 and 12:00 to minimize potential diurnal variation in enzyme activities (Thompson et al., 1988). Blood was collected by jugular venipuncture in heparinized 0.5 ml or 1.0 ml tuberculin syringes, transferred to heparinized microcentrifuge tubes, and kept on wet ice until centrifugation. Blood samples were centrifuged and plasma was separated from the cell pack and stored in microcentrifuge tubes at −20 C until analysis.

Brain tissue was removed from frozen bluebird carcasses, diluted with 0.1 M Tris buffer (pH 7.4, Sigma Chemical Company, St. Louis, Missouri, USA) and homogenized in teflon-on-glass homogenization tubes (Wheaton Instruments, Millville, New Jersey, USA). Starling brains were homogenized fresh using the same preparation procedure. The final dilution of bluebird and starling brains in buffer was 150 to 300-fold, depending on brain weight. Plasma was diluted 10-fold for starlings and 15-fold for bluebirds with 0.1 M Tris buffer (pH 7.4).

Brain and plasma samples were analyzed for ChE activity according to methods of Ellman et al. (1961), modified for use on a Vmax 96-well plate reader (Molecular Devices Corporation, Palo Alto, California, USA) set in kinetic mode at a wavelength of 405 nm with a run time of 2 min, read at 8 sec intervals, with 0 sec lag time, and with final volume of 250 µl/well. All samples were run in triplicate at 22 to 25 C. Optimal substrate (acetylthiocholine iodide (AThCh), Sigma Chemical Company) concentrations were determined for each species on non-study samples prior to analysis. Final concentrations were 8.0×10^{-4} M for AThCh, and 3.23×10^{-4} M for 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB, Sigma Chemical Company). Enzyme analysis included total brain and plasma ChE activity determinations. Plasma AChE activity was determined using the specific BChE inhibitor tetraisopropylpyrophosphoramide (iso-OMPA, Sigma Chemical Company) (Aldridge, 1953; Fairbrother et al., 1991) at a concentration of 10^{-4} M. Optimal iso-OMPA concentration was determined on non-study plasma samples prior to analysis to ensure complete BChE inhibition without AChE inhibition. Plasma BChE activity was computed as the difference between total ChE and AChE activity. All activities were converted from optical density units/minute to

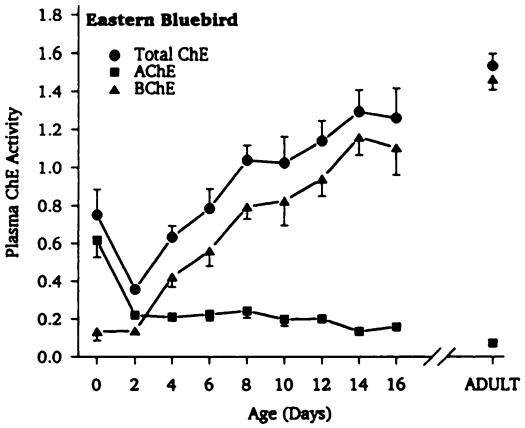

FIGURE 1. Plasma ChE activity (μmoles acetylthiocholine hydrolyzed/min/ml plasma ± SE) of eastern bluebird nestlings (n = 5 to 15 birds/age) and adults (n = 8) in relation to age.

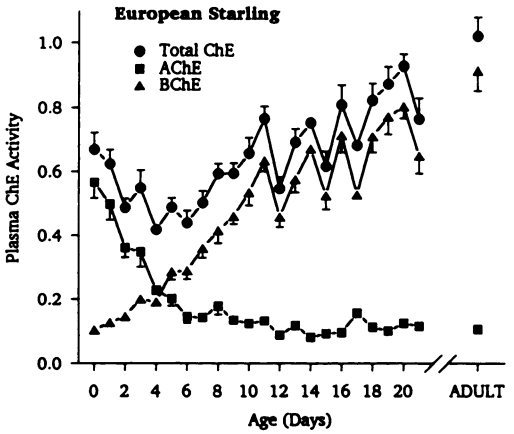

FIGURE 2. Plasma ChE activity (μmoles acetylthiocholine hydrolyzed/min/ml plasma ± SE) of European starling nestlings (n = 10 birds/age) and adults (n = 12) in relation to age.

μmoles AThCh hydrolyzed/min/ml plasma or g brain tissue.

Linear regressions (Zar, 1974) were used to determine the relationship between brain ChE activity and nestling age or brain weight. Comparisons of enzyme activity between ages or sexes were done using two-tailed t-tests (Zar, 1974). For all tests, a significance level of α = 0.05 was used.

RESULTS

Bluebird and starling nestlings had similar patterns of age-dependent plasma ChE activity (Figs. 1, 2). Acetylcholinesterase accounted for about 85% of total plasma ChE activity at day 0 in both species, but dropped sharply within 2 to 5 days posthatch, then remained constant throughout the remainder of the nestling stage. At fledging, AChE constituted 12 to 15% of total plasma ChE activity in both species. Plasma BChE activity increased throughout the nestling period, but was more variable in older starlings than in older bluebirds. Total ChE activity in adult bluebirds was 1.5 μmoles AThCh hydrolyzed/min/ml plasma, of which only 4.7% was attributable to AChE (Fig. 1). Butyrylcholinesterase activity was significantly higher and AChE activity was significantly lower in plasma of adult bluebirds than 16-day-old nestlings ($P < 0.05$). The difference in total plasma ChE activity between adults and 16-day-old bluebirds was not significant ($P > 0.05$). Based on the small sample size, there were no significant differences in enzyme activity between sexes for adult birds ($P > 0.10$). Total plasma ChE activity in adult starling was 1.0 μmoles AThCh hydrolyzed/min/ml plasma, of which AChE activity constituted 10.4% (Fig. 2). Total ChE and BChE activities were significantly ($P < 0.05$) higher in adult starling plasma than in 21-day-old birds, but differences in plasma AChE activity were not significant ($P > 0.50$). Plasma total ChE and BChE activities were significantly ($P < 0.001$) higher in adult bluebirds than in adult starlings, while plasma AChE activity was significantly ($P < 0.05$) higher in adult starlings.

Serial plasma samples drawn from four individual bluebird nestlings (three males, one female) had similar patterns of ChE activity as seen in singularly sampled birds (Fig. 3). However, since the first samples were not taken until birds were 3 days old, the steep declines in plasma AChE and total ChE activity from day 0 to 2 were not detected. All four birds had similar plasma ChE profiles.

Serial sampling of nestling bluebirds at 3 day intervals produced no deleterious

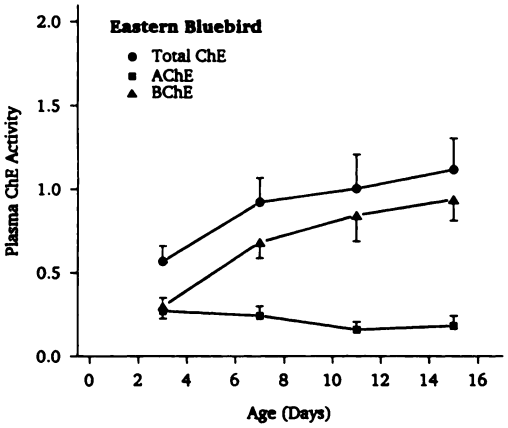

FIGURE 3. Plasma ChE activity (μmoles acetylthiocholine hydrolyzed/min/ml plasma ± SE) for sequential samples from four eastern bluebird nestlings, taken at four day intervals from 3 to 15 days old.

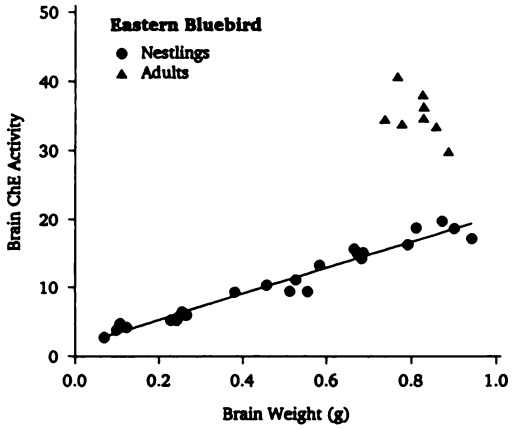

FIGURE 4. Brain ChE activity (μmoles acetylthiocholine hydrolyzed/min/g brain tissue) (Y) of eastern bluebird nestlings (Y = 1.49 + 19.07X, r = 0.98, n = 27, $P < 0.01$) and adults (n = 8) in relation to brain weight (X).

effects. All serially bled birds fledged. The mean (SD) weight of serially bled birds was 24.7 (1.7) g at 15 days old (1 to 2 days prior to fledging), while siblings which were not bled weighed 24.3 (1.3) g. The difference in body weights was not significant ($P > 0.60$). In most instances, no external signs of previous bleedings were apparent when nests were revisited for subsequent samples. Several nestlings had minor bruising at the site of venipuncture, but most bruises or hematomas disappeared within 2 days.

Brain ChE activity (Y) was significantly ($P < 0.01$) correlated with age (X) for nestling bluebirds (Y = 3.03 + 0.94X, r = 0.98, n = 27) and nestling starlings (Y = 5.30 + 0.64X, r = 0.92, n = 221). Slopes of the regression lines differed significantly ($P < 0.001$) between species, with the rate of increase of brain ChE activity with age being more rapid in bluebird nestlings than in starlings. Mean (SD) brain ChE activity in adult bluebirds was 35.2 (3.2) μmoles AThCh hydrolyzed/min/g tissue, which was 94% greater than in 16-day-old nestlings. The difference was highly significant ($P < 0.001$). Mean (SD) brain ChE activity in adult starlings (22.7 (1.9) μmoles AThCh hydrolyzed/min/g tissue) was 12% greater than in 21-day-old nestlings, which also was a highly significant difference ($P < 0.002$). Adult bluebirds had significantly higher brain ChE activity than adult starlings ($P < 0.001$). Brain ChE activity was correlated with brain weight in nestling bluebirds but not in adults (Fig. 4).

DISCUSSION

Bluebirds and starlings had similar age-dependent patterns of plasma AChE and BChE activity. Butyrylcholinesterase activity increased throughout the nestling stage, and also between fledging and sexual maturity. Following a rapid reduction immediately after hatching, plasma AChE activity remained at a constant, low level throughout the remainder of the nestling stage. Plasma AChE activity declined post-fledging, although this was more pronounced in bluebirds than in starlings. A nearly identical pattern of age-dependent changes in plasma enzyme activities has been found in altricial red-winged blackbirds (*Agelaius phoeniceus*) (M. Hooper, unpubl.); thus, the pattern may be common to a range of passerine species. Further research is required to determine if nestlings of other altricial species display similar patterns of enzyme activity.

Plasma ChE profiles documented here for altricial species contrast with those re-

ported previously for precocial mallards and chickens. Fairbrother et al. (1990) found decreasing total plasma ChE activity in growing mallards, and Bennett and Bennett (1991) later showed this to be due to concurrent decreases in both AChE and BChE activity. Lyles et al. (1980) detected a rapid decrease in AChE activity in chicken plasma immediately after hatch, with a simultaneous increase in BChE activity. Bennett and Bennett (1991) did not observe a rapid initial decrease in plasma AChE activity, and the percentage of total plasma ChE activity attributable to AChE was essentially equal at all ages. Since their first samples were taken when birds were 7 days old, any decrease in mallard plasma AChE activity which might occur shortly after hatching would not have been detected.

Plasma ChE activities at hatching are a continuation of trends detected in the embryo (Lyles et al., 1980; Smucker and Wilson, 1990). The liver is considered the source of plasma BChE, since developmental increases in plasma BChE activity coincide with a decline in liver BChE activity, suggesting that liver BChE is being mobilized and released (Smucker and Wilson, 1990). The origin of plasma AChE is not known, since little is found in liver homogenates. However, developing muscle and brain contain high AChE activity suggesting extrahepatic sources for this enzyme (Smucker and Wilson, 1990). Further research is required to determine the cause of rapid decreases in plasma AChE activity immediately after hatch in many avian species.

Brain ChE activity in nestling starlings was found to be age-dependent, as has been previously reported (Grue et al., 1981; Robinson et al., 1988). Eastern bluebird nestlings had a similar pattern of age-dependent increases in brain ChE activity as found in other altricial species (Grue et al., 1981; Custer and Ohlendorf, 1989). Based on the adult bluebirds collected as controls, brain ChE activity in fledglings appeared to be about half the adult value. However, the age at which adult ChE levels were attained cannot be estimated from the linear regression equation given since development of brain ChE activity apparently follows a sigmoidal pattern (Grue and Hunter, 1984). Brain ChE activity of bluebirds at a series of known ages between 16 days and 1 yr of age is required to accurately model the pattern of development in this species.

Age-dependent sensitivity of passerines to ChE-inhibiting OP compounds has been documented in experiments where European starlings have been exposed to dicrotophos and diazinon (Grue and Shipley, 1984; Hooper et al., 1990). Similar age-dependent sensitivity has been found in eastern bluebirds dosed with diazinon (N. Gard and M. Hooper, unpubl.). The first few days after hatching is the most critical period since newly hatched birds are much more sensitive to OP's than are adults, and nestlings are susceptible to ChE inhibition either through direct exposure to OP compounds or consumption of contaminated food. Age-dependent differences in sensitivity to OP's might arise since nestlings have less ChE per gram brain weight than adults (Fig. 4), and possibly a less well developed blood-brain barrier (Bolton, 1976). Additionally, since plasma BChE usually is inhibited more rapidly and to a larger degree than brain ChE (Hill and Fleming, 1982), BChE may be scavenging the active oxon forms of OP compounds that otherwise might inhibit brain AChE activity (Gupta and Dettbarn, 1987). A similar role has been demonstrated for plasma carboxyesterases in rats, which act as an alternative target to sequester significant amounts of the oxon form of parathion before it is able to inhibit brain AChE (Chambers and Chambers, 1990). Since plasma BChE activity increases with age in nestling passerines, this might partially account for decreasing sensitivity in older birds. Because of the lack of OP hydrolyzing enzymes in the plasma of many bird species (Mackness et al., 1987) or the low affinity of this class of enzymes for OP's

in birds (Landis and Shough, in press), the role of such plasma cholinesterases as BChE in protecting individuals becomes more important.

In summary, age dependent changes in plasma ChE activities occurred in two altricial species, but the patterns were not identical to those described for precocial species. Thus, evaluations of exposure to ChE-inhibiting pesticides must be made using same-aged individuals, and the results from precocial young should not be extrapolated to altricial species. Studies designed to monitor nestling exposure to ChE inhibitors will require baseline data on age-dependent plasma AChE and BChE activities in unexposed birds, and a means of reliably aging birds.

ACKNOWLEDGMENTS

We thank Tracy Hannaford, Ron Kroner, Thomas Martin, and Marti Wolfe for performing the Washington field work and ChE analyses; Linda Carlock, Katherine Hunt, and Sherry Skipper for assisting in ChE analyses; Dale Droge and Jon Plissner for use of their bluebird nest box colony; Stephen Eakes and Richard White for permitting establishment of bluebird nest boxes on their properties. We are grateful to Ronald J. Kendall for providing much of the impetus for the initial development and direction of this study, and to Richard Balcomb and the Ciba-Geigy Corporation for supporting this research.

LITERATURE CITED

ALDRIDGE, W. N. 1953. The differentiation of true and pseudo cholinesterase by organophosphorus compounds. Biochemical Journal 53: 62–67.

BENNETT, R. S., AND J. K. BENNETT. 1991. Age-dependent changes in activity of mallard plasma cholinesterases. Journal of Wildlife Diseases 27: 116–118.

BOLTON, T. B. 1976. Nervous system. *In* Avian physiology, P. D. Sturkie (ed.). Springer-Verlag, New York, New York, pp. 1–28.

CHAMBERS, J. E., AND H. W. CHAMBERS. 1990. Time course of inhibition of acetylcholinesterase and aliesterases following parathion and paraoxon exposures in rats. Toxicology and Applied Pharmacology 103: 420–429.

CUSTER, T. W., AND H. M. OHLENDORF. 1989. Brain cholinesterase activity of nestling great egrets, snowy egrets and black-crowned night-herons. Journal of Wildlife Diseases 25: 359–363.

ELLMAN, G. L., K. D. COURTNEY, V. ANDRES. JR., AND M. R. FEATHERSTONE. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochemical Pharmacology 7: 88–98.

FAIRBROTHER, A., R. S. BENNETT, AND J. K. BENNETT. 1989. Sequential sampling of plasma cholinesterase in mallards (*Anas platyrhynchos*) as an indicator of exposure to cholinesterase inhibitors. Environmental Toxicology and Chemistry 8: 117–122.

———, M. A. CRAIG, K. WALKER, AND D. O'LOUGHLIN. 1990. Changes in mallard (*Anas platyrhynchos*) serum chemistry due to age, sex, and reproductive condition. Journal of Wildlife Diseases 26: 67–77.

———, B. T. MARDEN, J. K. BENNETT, AND M. J. HOOPER. 1991. Methods used in determination of cholinesterase activity. *In* Cholinesterase-inhibiting insecticides, P. Mineau (ed.). Elsevier, Amsterdam, Netherlands, pp. 35–71.

FARAGE-ELAWAR, M. 1991. Development of esterase activities in the chicken before and after hatching. Neurotoxicology and Teratology 13: 147–152.

GRUE, C. E., AND C. C. HUNTER. 1984. Brain cholinesterase activity in fledgling starlings: Implications for monitoring exposure of songbirds to ChE inhibitors. Bulletin of Environmental Contamination and Toxicology 32: 282–289.

———, AND B. K. SHIPLEY. 1984. Sensitivity of nestling and adult starlings to dicrotophos, an organophosphate pesticide. Environmental Research 35: 454–465.

———, G. V. N. POWELL, AND N. L. GLADSON. 1981. Brain cholinesterase (ChE) activity in nestling starlings: Implications for monitoring exposure of nestling songbirds to ChE inhibitors. Bulletin of Environmental Contamination and Toxicology 26: 544–547.

GUPTA, R. C., AND W.-D. DETTBARN. 1987. Iso-OMPA-induced potentiation of soman toxicity in rat. Archives of Toxicology 61: 58–62.

HILL, E. F., AND W. J. FLEMING. 1982. Anticholinesterase poisoning of birds: Field monitoring and diagnosis of acute poisoning. Environmental Toxicology and Chemistry 1: 27–38.

HOFFMAN, D. J., AND W. C. EASTIN, JR. 1981. Effects of malathion, diazinon, and parathion on mallard embryo development and cholinesterase activity. Environmental Research 26: 472–485.

HOOPER, M. J., L. W. BREWER, G. P. COBB, AND R. J. KENDALL. 1990. An integrated laboratory and field approach for assessing hazards of pesticide exposure to wildlife. *In* Pesticide effects on terrestrial wildlife, L. Somerville and C. H. Walker (eds.). Taylor & Francis, London, England, pp. 271–283.

LANDIS, W. G., AND N. J. SHOUGH. 1992. Discovery, initial characterization and comparison of the organophosphate acid hydrolyzing activities of the bobwhite quail, stilt and mallard. Comparative Biochemistry and Physiology 102(C): 527–535.

LUDKE, J. L., E. F. HILL, AND M. P. DIETER. 1975. Cholinesterase (ChE) response and related mortality among birds fed ChE inhibitors. Archives of Environmental Contamination and Toxicology 3: 1–21.

LYLES, J. M., E. A. BARNARD, AND I. SILMAN. 1980. Changes in levels and forms of cholinesterases in blood plasma of normal and dystrophic chickens. Journal of Neurochemistry 34: 978–987.

MACKNESS, M. I., H. M. THOMPSON, A. R. HARDY, AND C. H. WALKER. 1987. Distinction between 'A' esterases and aryl esterases: Implications for esterase classification. Biochemical Journal 245: 293–296.

ROBINSON, S. C., R. J. KENDALL, R. ROBINSON, C. J. DRIVER, AND T. E. LACHER, JR. 1988. Effects of agricultural spraying of methyl parathion on cholinesterase activity and reproductive success in wild starlings (*Sturnus vulgaris*). Environmental Toxicology and Chemistry 7: 343–349.

SMUCKER, S. J., AND B. W. WILSON. 1990. Multiple molecular forms and lectin interactions of organophosphate-sensitive plasma and liver esterases during development of the chick. Biochemical Pharmacology 40: 1907–1913.

THOMPSON, H. M., C. H. WALKER, AND A. R. HARDY. 1988. Avian esterases as indicators of exposure to insecticides—The factor of diurnal variation. Bulletin of Environmental Contamination and Toxicology 41: 4–11.

WESTLAKE, G. E., P. J. BUNYAN, A. D. MARTIN, P. I. STANLEY, AND L. C. STEED. 1981. Organophosphate poisoning. Effects of selected organophosphate pesticides on plasma enzymes and brain esterases of Japanese quail (*Coturnix coturnix japonica*). Journal of Agriculture and Food Chemistry 29: 772–778.

WHITE, D. H., K. A. KING, C. A. MITCHELL, E. F. HILL, AND T. G. LAMONT. 1979. Parathion causes secondary poisoning in a laughing gull breeding colony. Bulletin of Environmental Contamination and Toxicology 23: 281–284.

ZAR, J. H. 1974. Biostatistical analysis. Prentice-Hall, Inc., Englewood Cliffs, New Jersey, 620 pp.

Received for publication 17 January 1992.