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DEVELOPMENT OF REFERENCE RANGES FOR PLASMA TOTAL CHOLINESTERASE AND BRAIN ACETYLCHOLINESTERASE ACTIVITY IN FREE-RANGING CARNABY'S BLACK-COCKATOOS (*CALYPTORHYNCHUS LATIROSTRIS*)

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ABSTRACT: Published avian reference ranges for plasma cholinesterase (ChE) and brain acetylcholinesterase (AChE) are numerous. However, a consistently reported recommendation is the need for species- and laboratory-specific reference ranges because of variables, including assay methods, sample storage conditions, season, and bird sex, age, and physiologic status. We developed normal reference ranges for brain AChE and plasma total ChE (tChE) activity for Carnaby's Black-Cockatoos (*Calyptorhynchus latirostris*) using a standardized protocol (substrate acetylthiocholine at 25 C). We report reference ranges for brain AChE (19–41 μmol/min per g, mean 21±6.38) and plasma tChE (0.41–0.53 μmol/min per mL, mean 0.47±0.11) (n=15). This information will be of use in the ongoing field investigation of a paresis-paralysis syndrome in the endangered Carnaby's Black-Cockatoos, suspected to be associated with exposure to anticholinesterase compounds and add to the paucity of reference ranges for plasma tChE and brain AChE in Australian psittacine birds.
Key words: Anticholinesterase compounds, black cockatoos, psittacine, reference ranges.

INTRODUCTION

Three species of black cockatoo are endemic to the southwest of Western Australia. Carnaby's Black-Cockatoo (*Calyptorhynchus latirostris*) is threatened and listed as "endangered" under the Australian Government's *Environment Protection and Biodiversity Conservation Act 1999* (Department of Parks and Wildlife 2013). Baudin's Cockatoo (*Calyptorhynchus baudinii*) and the forest Red-tailed Black Cockatoo (*Calyptorhynchus banksii naso*) are classified as "vulnerable" under federal legislation. All three species are listed as "rare or likely to become extinct" under Western Australian legislation. The principal factors that have caused decline in these species include habitat loss, competition for nest hollows by other birds and feral honeybees, vehicle strike, and illegal shooting by landowners to protect fruit and nut crops (Mawson and Johnstone 1997; Department of Parks and Wildlife 2013).

The Perth Zoo Veterinary Department (PZVD), on behalf of the Western Australian Department of Parks and Wildlife, receives sick and injured black cockatoos from across Western Australia for triage, treatment, and supportive care. From 30 January to 8 July 2012, 21 Carnaby's Black-Cockatoos were presented to the PZVD with varying degrees of paresis (n=21), without signs of primary trauma. Toxicosis from organophosphate and carbamate insecticides was high on the differential diagnosis list on the basis of clinical signs and recent reports of fenthion (an organophosphate) toxicity in three Little Corella (*Cacatua sanguinea*) die-off events in the vicinity (Wildlife Health Australia 2012, unpubl. data). Other differential diagnoses included trauma, heavy metal exposure, other toxicants (organochlorine and pyrethroid insecticides, anticoagulant rodenticides), neon-

icitinoids, botulism, starvation/nutritional deficiency, and infectious diseases. These differentials were less consistent with the clinical presentation and were not supported by histopathologic evidence (K. Warren unpubl. data).

Cholinesterase (ChE) inhibiting compounds, including organophosphate (OP), and carbamates are used routinely throughout Australia for agricultural pest control. Organophosphate and carbamate pesticides disrupt the nervous system through the inhibition of esterases, including acetylcholinesterase (AChE) and butyrylcholinesterase (BChE, also known as pseudocholinesterase). These enzymes catalyze the hydrolysis of the neurotransmitter acetylcholine in the central and peripheral nervous systems but differ in substrate specificity, inhibitor susceptibility, and tissue distribution (Thompson and Walker 1994; Radic and Taylor 2006). Exposure of birds and mammals to these agents can induce acute toxicosis, with signs including incoordination, weakness, ataxia, muscle tremor, diarrhea, convulsions, respiratory difficulty, and bradycardia (Shimsoni et al. 2012). Exposure to high levels of these insecticides may result in paralysis and fatal respiratory failure (Shimsoni et al. 2012). Minimal experimental data exists on the sensitivity of Australian psittacine birds to OPs such as fenthion, with only one report available in the Galah (*Eolophus roseicapilla*), which was found to be highly sensitive to fenthion ethyl (LD50, 4.5 mg/kg; McIlroy 1985).

Diagnosis of insecticide toxicosis in wildlife often relies on detection of insecticide residues in alimentary tract contents. However, this method is hampered in wild birds by autolysis of carcasses, lack of availability of ingesta, insecticide concentrations below detection limits, or exposure through the dermal route rather than ingestion (Shimsoni et al. 2012). More recently, the measurement of blood or tissue ChE activity has been used to diagnose OP or carbamate insecticide toxicosis in animals (Fairbrother et al. 1991; Fairbrother 1996; Fildes et al. 2006, 2009; Shimsoni et al. 2012). If brain AChE activity is inhibited by 20% from normal, exposure to an anticholinesterase compound is considered likely; however, the accepted diagnostic convention is that whole-brain AChE inhibition >50% is indicative of anti-ChE toxicosis (Ludke et al. 1975; Hill and Fleming 1982).

Wide interspecies differences exist in avian plasma levels of AChE and other esterases (Roy et al. 2005). It is recommended that species-specific reference ranges be used when attempting to diagnose anticholinesterase exposure and toxicoses (Hill 1988; Shimsoni et al. 2012). In Australia little information is published on plasma ChE activity in native birds, other than by Fildes et al. (2009), and no existing reference ranges for Australian psittacine birds other than the Budgerigar (*Melopsittacus undulatus*). Given this diagnostic gap, we developed reference ranges for normal brain AChE and plasma total ChE (tChE) activity in Carnaby's Black-Cockatoos, using a standardized protocol, to enable informed interpretation of results in the diagnosis of black cockatoo mortality events.

MATERIALS AND METHODS

Fifteen brain samples and 15 blood samples were collected from free-ranging Carnaby's Black-Cockatoos of both sexes presented to PZVD between January 2013 and March 2014. Cockatoos were submitted to the PZVD by wildlife rehabilitators, Department of Parks and Wildlife rangers, or members of the public. Birds were of postfledging age, with history and postmortem findings consistent with acute trauma (vehicle strike or shotgun injury). It is not unlikely that birds included in this study came from the same populations as birds clinically affected by the aforementioned paralysis syndrome and therefore may have been exposed to environmental chemicals at some point. However, clinically affected birds had a very specific presentation of bilateral hind limb paresis or paralysis, whereas individuals used in this study did not present in this clinical manner and had no signs of bilateral hind limb paresis or paralysis, which might otherwise have suggested the possibility of recent clinically significant exposure to anticholinesterases. Birds were euthanatized because of the poor prognosis for recovery as assessed on clinical examination.

On initial examination, heart rate, body condition score (BCS) ranging from 0 (emaciated) to 3 (obese), weight, withdrawal responses, and tone of the digits, legs, and cloaca were assessed. Birds

were also examined for evidence of chronic paresis, such as bruising and crust formation to the keel and soiling around the cloaca. A comprehensive physical examination under anesthesia was undertaken during the first 48 h after presentation and included radiographs to diagnose traumatic injury.

Two milliliters of whole blood was collected from the right jugular vein. Packed cell volume (PCV) and total protein (TP) were measured so that corrections for dehydration could be made when determining plasma tChE. Hydration status was measured through analysis of PCV and TP then compared with normal reference intervals for these parameters (Le Souef et al. 2013).

After venipuncture, 0.1 mL was placed into two microhematocrit tubes, centrifuged at room temperature (1,370 × G for 5 min) for PCV and TP determination (via microhematocrit and refractometry). Residual volume was placed into ethylenediaminetetraacetic acid (EDTA) chilled and forwarded to the Department of Food and Agriculture (DAFWA), South Perth, Western Australia, for centrifugation, duplicate PCV and TP evaluation, and plasma extraction. Packed cell volume and TP evaluation was undertaken when the samples arrived at DAFWA, and the remaining plasma was stored for up to 18 mo at –80 C until completion of the sample set. Plasma samples were the assayed for tChE in a single analytical run to minimize handling and assay procedure variance. After euthanasia, the whole body was chilled immediately, and the brain was dissected within 24 h of death. The brain was incised longitudinally into two sections, which were weighed then stored in plastic ziplock bags at –18 C then forwarded to DAFWA. Postmortem examination and collection of tissue samples for possible future histopathology were also undertaken. Ventriculus and crop contents were collected and stored frozen at –20 C for possible future residue analysis, because sublethal exposure to ChE inhibitors may make animals more vulnerable to collision with objects (Mineau and Tucker 2002), potentially confounding results. To eliminate interassay variability, all brain samples were analyzed in a single analytical run. Acetylcholinesterase activities of brain and plasma tChE were undertaken at DAFWA and determined colorimetrically by the Ellman assay (Ellman et al. 1961) as described by Wilson and Henderson (2007). In this assay the substrate for AChE, acetylthiocholine iodide (ATCh), is incubated with the sample and the color reagent, 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), which reacts with sulfhydryl groups to form a yellow-colored anion, 5-thio-2-nitrobenzoate (TNB). The thiocholine produced by the enzymatic hydrolysis of ATCh reacts with DTNB to produce TNB. The rate of formation of TNB can be determined colorimetrically.

Both brain and plasma samples were stored at –80 C until analyzed. One half of the brain (cut longitudinally) was homogenized in chilled 0.1 M buffer with 1% Triton X-100 (pH 8.0) using an Ultra Turrax homogenizer (Sigma-Aldrich, Castle Hill, New South Wales, Australia) at low speed. The ratio of tissue to buffer was about 1:40. The homogenate was centrifuge at 1,000 × G for 10 min. Before the colorimetric assay, brain homogenates and EDTA plasma were diluted 1:10 in Milli-Q water (Merck Millipore, Bayswater, Victoria, Australia). The colorimetric assay was performed in 96-well plates. The assay mix consisted of 250 µL of 0.1 M phosphate buffer (pH 8), 10 µL of 10.3 mM DTNB D8130 (Sigma-Aldrich), and 30 µL of sample, and the reaction was started with 30 µL of ATCh A5751 (Sigma-Aldrich). The reaction rate was monitored at 412 nm on a POLARstar Omega (BMG Labtech, Mornington, Victoria, Australia) at 25 C. Sample blanks (exclude ATCh) were included to measure free sulfhydryls in each sample, as well as a substrate blank (excludes sample) to measure nonenzymatic hydrolysis of ATCh. All samples were run in duplicate. A calculation factor was established for the POLARstar using known concentrations of free sulfhydryl (L-glutathione-reduced G4251; Sigma-Aldrich) under conditions of the assay in the absence of sample. Pooled plasma and brain homogenate were used for quality control. These pools were validated against a commercial source of enzyme (AChE from *Electrophorus electricus* [electric eel] C3389; Sigma-Aldrich).

Statistical analysis was performed using SPSS (version 22, Chicago, Illinois, USA). Because the sample size was small (>40), suggested reference ranges include a range of values (minimum to maximum), rather than a range developed from mean±2 SD (Geffré et al. 2009). Normality of the data was determined using the Shapiro-Wilk test. For those normally distributed data, the mean, SD, minimum, maximum, and 95% confidence levels were computed. For data with a nonparametric distribution, the median, minimum, maximum and 10–90 percentiles were computed.

RESULTS

Nine females and six males were sampled. Eight of the 15 samples were paired brain and plasma samples; the remaining were individual samples. The mean BCS of birds sampled for plasma tChE was 1.46±0.35. The mean BCS of birds sampled for brain AChE was

TABLE 1. Reference ranges for brain acetylcholinesterase (AChE) and plasma total cholinesterase (tChE) (Ellman method, substrate acetylthiocholine at 25 C) in 15 Carnaby's Black-Cockatoos (*Calyptorhynchus latirostris*) in Australia. CL = confidence level.

Analytes	Mean±SD	Standard error	Upper 95% CL for mean	Lower 95% CL for mean	Minimum	Maximum
Plasma tChE (μmol/min per mL)	0.47±0.11	0.02	0.53	0.41	0.28	0.65
Brain AChE (μmol/min per g)	21±6.38	1.05	24.53	17.53	19	41

1.46±0.42. The mean weight of the birds sampled for plasma tChE reference intervals was 508 g (range 402–618 g), and the mean weight of the birds sampled for brain AChE reference intervals was 509 g (range 320–618 g).

Suggested reference ranges calculated for brain AChE and plasma tChE for Carnaby's Black-Cockatoos are shown in Table 1. Brain AChE range was 19–41 μmol/min per g (mean 21); *n*=15). Plasma tChE range was 0.41–0.53 μmol/min per mL (*n*=15). Therefore 50% depression of our calculated brain AChE reference range (and probable toxicoses) would equate to 21.7 μmol/min per g (range 9.5–20.5). Twenty percent depression (and probable exposure) would equate to 34.8 μmol/min per g (range 15.2–32.8). Fifty percent depression of our calculated tChE reference range (and probable toxicoses) would equate to 0.235 μmol/min per mL (range 0.2–0.26). Twenty percent depression (and probable exposure) would equate to 0.37 μmol/min per mL (range 0.32–0.42).

Hydration status, measured through analysis of PCV and TP, showed three of the 15 individuals were clinically dehydrated and had PCV and TP values above normal reference intervals (PCV=0.45±0.03 L/L and TP=29.3±2.1 g/L; Le Souef et al. 2013). One of these individuals had an outlier plasma tChE value and was discarded from the reference interval study.

DISCUSSION

Published avian reference ranges for plasma ChE and brain AChE are numerous (Westlake et al. 1981; Hill 1988; Gard and Hooper 1993; Tully et al. 2003; Fildes et al. 2009; Santos et al. 2012; Shimsoni et al. 2012; Grossett et al. 2014). However, a consistently reported recommendation is the need for species-specific and laboratory-specific reference ranges (Hill 1988; Shimsoni et al. 2012). Westlake et al. (1983) examined brain AChE in 47 bird species and plasma AChE in 19 species and demonstrated family trends despite interspecies variation. Too few native Australian bird species reference ranges have been established to identify phylogenetic trends (Fildes et al. 2009), and the few reported values in psittacine birds worldwide are highly variable. The mean AChE activity in the Hispaniolan Amazon Parrot (*Amazona ventralis*) (0.48 μmol/min per mL), using the same laboratory methodology as the current study, is similar to the mean of 0.47 μmol/min per mL determined for Carnaby's Black-Cockatoos (Tully et al. 2003). However, the mean AChE activity in the Budgerigar (89 μmol/min per mL) was well above the mean for Carnaby's Black-Cockatoos despite a similar methodology (Fildes et al. 2009). Such variation highlights the need for species-specific reference values and minimizing variation in test methodology to aid field investigation of toxicity events.

Plasma AChE and BChE are both valid markers of OP-related exposure, and both give early warning of exposure before adverse clinical health. Plasma AChE activity is thought to approximate neuronal AChE more closely than BChE, although both have advantages and disadvantages in particular situations (e.g., biomonitoring compared with toxicity) (Kapka-Skrzypczak et al. 2011). In birds, plasma BChE is often inhibited more rapidly and to a larger degree than brain AChE by low-level exposure to OPs and

carbamates, hence its use in humans as a biomonitoring tool. Butyrylcholinesterase may also scavenge the active forms of ChE-inhibiting compounds that might otherwise inhibit brain AChE activity (Lumeij 2008). However, there are difficulties in interpretation. Inhibition of BChE is less clearly correlated with clinical signs of toxicity compared with AChE activity (Hart 1993; Fildes et al. 2009). Plasma BChE recovers rapidly after a single exposure, so exposure may be "missed," especially because frequent blood sampling is often not practical, especially in wild bird populations. However, for a hospitalized bird undergoing treatment, repeated measurement of plasma ChE offers a reliable indication of exposure to an OP ChE inhibitor if serial samples show a recovery to normal levels over time (Mineau and Tucker 2002). In contrast to OP poisoning, carbamates often can produce false negatives because of reactivation of AChE. This is especially true for field samples in which time and temperature can result in a significant increase in AChE activity. This reactivation can be fast; for example, the half-life for recovery of *N*-methylcarbamylated AChE is approximately 30 min (Fukuto 1990). This recovery may partially mask a lethal pesticide toxicosis (Smith et al. 1995). It is always recommended that results of ChE, AChE, and BChE be compared with results of nonexposed individuals of the same species and age, because results are not interchangeable between species (Lumeij 2008).

In a wildlife disease investigation in 2012, nine Little Corellas were found weak or dead at a park in the Perth metropolitan area with suspected OP poisoning. Measurement of brain AChE revealed decreased levels (range 1.3–10.2 μmol/min per g), and fenthion was detected in the gut contents of one bird (53 mg/kg, ChemCentre, Bentley, Western Australia, Australia) (Wildlife Health Australia 2012, unpubl. data). This example provides an interesting and consistent comparison to our generated reference ranges, analyzed using the same methods and at the same laboratory in likely the most closely related Australian cockatoo species where toxicology data is available.

In our reference range study, three birds were assessed as clinically dehydrated, one of which had a plasma tChE activity above suggested reference intervals. Heffernan et al. (2012) reported that dehydration caused a significant increase in plasma ChE activity. In their study in quail (*Coturnix coturnix*), the combination of dehydration and the OP dicrotophos exposure produced plasma ChE activity that was not significantly different from reference and pre-exposure values, confounding the diagnosis of anticholinesterase exposure. A larger sample size would be required to explore and validate such findings further in relation to Carnaby's Black-Cockatoos. Clinicians responsible for diagnosis of anticholinesterase exposure in birds should be aware that dehydration may mask the effect of anticholinesterases on plasma ChE activity, that methods to adjust plasma ChE activities for the confounding effects of dehydration have been developed, and that these methods will facilitate the diagnosis of anticholinesterase exposure in dehydrated, exposed birds (Heffernan et al. 2012).

Ideally it would be advantageous to increase sample size in the future. Based on the distribution of birds during the breeding season, the total population of Carnaby's Black-Cockatoo has been estimated at between 11,000 and 60,000 birds (Saunders et al. 1985). Garnett and Crowley (2000) consider an estimate of 60,000 breeding birds to be of medium reliability. However, it has also been claimed that the population could number <10,000 birds (Mawson and Johnstone 1997). It is difficult if not impossible to sample wild birds in the field other than at breeding time when they inhabit tree hollows, so we used the accessible population size of those brought in to the PZVD for triage for our sampling. On the basis of PZVD records from 2009 to 2014, the mean number of Carnaby's Black-Cockatoos presented to the PZVD is 135 birds/yr. Power analysis was undertaken based on the mean and SD of population size estimated from the literature and the accessible population size. Results of

power analysis indicated we needed to sample eight individuals to assure adequate power to detect sample significance (Kane 2015) at an alpha (probability of type I error) of 0.05 and 90% power (most medical literature uses a power of 80–90% (beta 0.1–0.2). However, we received funding that allowed us to sample 15 individuals, and this larger sample size was chosen because of the uncertainty of the true population size.

The development of reference ranges for plasma tChE and brain AChE is crucial to the ongoing investigation of the role of anticholinesterase exposure as a cause for paresis in the endangered Carnaby's Black-Cockatoos. Ongoing evaluation of plasma tChE and brain AChE against reference ranges in affected birds will also assist in differentiating any future anticholinesterase toxicity events.

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