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Characterization of plasma cholinesterase from the White stork (*Ciconia ciconia*) and its *in vitro* inhibition by anticholinesterase pesticides

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ABSTRACT

Blood plasma cholinesterase (ChE) activity is a sensitive biomarker of exposure to organophosphorus (OP) and carbamate (CB) insecticides in vertebrates. Several studies indicate that more than one ChE form may be present in blood of birds. In this study the predominant ChE activity (acetylcholinesterase – AChE– or butyrylcholinesterase – BChE–), the range of ChE activity as well as ChE age-dependent changes in non-exposed individuals of White stork (*Ciconia ciconia*) have been established. The *in vitro* sensitivity of ChE to OP and CB insecticides such as paraoxon-methyl, carbofuran and carbaryl was also investigated. Plasma ChE was characterised using three substrates (acetylthiocholine iodide, propionylthiocholine iodide, and S-butyrylthiocholine iodide) and three ChE inhibitors (eserine sulphate, BW284C51 and iso-OMPA). The results indicated that propionylthiocholine was the preferred substrate by plasma cholinesterase followed by acetylcholine and butyrylcholine and the predominant enzymatic activity in plasma of White storks was BChE. Normal plasma BChE activity in White stork was 0.32 ± 0.01 μmol/min/ml for adults and 0.28 ± 0.03 μmol/min/ml for juveniles. So, the age had no significant effect on the range of BChE activity. The study on the *in vitro* inhibitory potential of tested anticholinesterase pesticides on plasma ChE activity revealed that paraoxon-methyl is the most potent inhibitor followed by carbofuran and finally by carbaryl. The percentage of *in vitro* plasma ChE inhibition was observed to be similar between adults and juveniles.

1. Introduction

The White stork (*Ciconia ciconia*) is included in the IUCN Red List of Threatened Species as of "Least Concern". It is a widespread species in Europe with both resident and migrant populations in the Iberian Peninsula. According to Molina (2005), regions located in the Southwest of Spain, such as Extremadura, host the largest colonies. This emphasises the importance of its protection in our country. The knowledge on the impact of long-term exposure to pollution in wild populations is still scarce, probably due to difficulties in working with this species, particularly in that concerning to the collection of non-invasive samples in wild animals. However, in relation to its conservation in the Iberian Peninsula, these studies are needed since this is a long-living species (up to 33 years) (del Hoyo et al., 1992) and a considerable number of colonies are established in agricultural areas. These birds can be chronically exposed to agrochemical contamination in the case of resident populations or they can be exposed for several months, usually during the breeding season, in the case of migratory populations. Exposure to pesticides which occurs mainly through the ingestion of contaminated preys (mainly fish, amphibian, earthworms and snails) (Pinowski et al., 1991), seeds treated with pesticides or poisoned bait, inhalation and contact with contaminated air, soil and water is of particular concern due to the toxic properties of these chemicals. Extremadura is mainly an agricultural region with important natural spaces and therefore it offers a suitable habitat for these birds, which are largely resident. Pollutants present in their tissues and blood are mainly due to local sources of exposure, mainly to the pesticides used in agricultural activities or the presence of poisoned bait in the field. Thus, in addition to the high interest of assessing the risks for local populations inherent to their exposure to pesticides, the White stork is also a convenient bioindicator of the quality of these

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ecosystems since it is very abundant all through the region (Molina, 2005) where it lives all through the year because the most of populations are resident (Alonso et al., 1992).

Agricultural pesticides are known to compromise bird survival contributing to declining populations (Mineau, 2005), and it is possible that pesticides might be a contributing factor to the decline in storks in some locations. Organophosphorous (OP) and carbamate (CB) pesticides are commonly used in agricultural practices as insecticides because they are more degradable and have less persistence in the environment than organochlorine pesticides and in some occasion they are also used to make poisoned baits to fight against vermin into hunting or livestock areas. Consistently, they are of ecological concern since they are toxic to wild non-target species such as raptors (Hooper et al., 1989; Wilson et al., 1991; Goldstein et al., 1999). The toxicity of these pesticides is mainly due to the inhibition of acetylcholinesterase (AChE) activity, the enzyme which degrades the neurotransmitter acetylcholine in cholinergic synapses. The inhibition of AChE provokes an accumulation of acetylcholine at the nerve synapses and disruption of the nerve function (Peakall, 1992) a toxicity mechanism that may lead to mortality. The inhibition of cholinesterases (ChEs) is appropriate for evaluation of exposure to OP and CB pesticides in avian because these are rapidly degraded and excreted of the organisms and therefore they are not easily detectable by chemical analysis (Hill and Fleming, 1982; Hill, 1995; Fairbrother, 1996). Blood (ChEs), including AChE and a less specialized enzyme commonly designed by pseudocholinesterase or butyrylcholinesterase (BChE), are also inhibited by these substances being a widely used non-destrutive biomarker to diagnose the exposure of anticholinesterase agents (Sanchez et al., 1997). On the other hand, carboxilesterases are a group of non-specific esterase enzymes which can also be present in the plasma of birds and to be inhibited by these groups of substances (Thompson, 1999).

Since birds are very sensitive to OP and CB pesticides and are frequently key species in their ecosystems, blood ChE activity has been widely used to assess the exposure and effects of these agrochemicals in populations inhabiting agricultural areas (Westlake et al., 1981a, b; Gard and Hooper, 1993; Soler-Rodríguez et al., 1998; Parsons et al., 2000; Mayack and Martin, 2003; Rendón-von Osten et al., 2005; Roy et al., 2005). The results obtained showed the suitability of birds ChE as a biomarker for OP and CB pesticides. In birds, both AChE and BChE are found in blood plasma with wide interspecies differences (Walker and Thompson, 1991; McInnes et al., 1996). For both ethical and conservational reasons, the use of biomarkers to investigate the exposure to pollutant in populations of the White stork is mostly adequate since they can be determined in a non-destructive way and in some occasions provide early indications of toxic effects. Blood is in fact the best biological material for non-destructive biomarker analysis (Fossi et al., 1994). Plasma cholinesterase activities have been used to monitor exposure to anticholinesterase pesticides in many bird species (Dieter, 1975; Dieter and Ludke, 1975; Ludke et al., 1975; Westlake et al., 1981a,b; Hooper et al., 1989; Wilson et al., 1991; Rainwater et al., 1995; Goldstein et al., 1999; Parsons et al., 2000; Parker and Goldstein, 2000), but to the best of our knowledge there is not similar studies in White storks. Changes in the activity of cholinesterase enzymes circulating in the plasma observed in these studies have been considered by those authors as an indirect measure of pesticide exposure and residue accumulation. Before ChE being used as biomarker in biomonitoring studies with wild populations and to avoid bias in the interpretation of results it is important to characterise the enzymes present in the species and tissue to be analysed and to know the range of variability in non-exposed individuals (Thompson, 1999; García et al., 2000). Therefore, objectives of the current study were:

- to characterise the ChE enzymes present in the plasma of the White stork from wild populations of the Extremadura region (Spain) by studying their activity towards different substrates (acetylthiocholine, propyonilthiocholine and buthyrylthiocholine) and their sensitivity to selective inhibitors (eserine sulphate as inhibitor of all ChE enzymes, iso-OMPA as inhibitor of BChE and BW284C51 as inhibitor of AChE in vertebrates).
- to determine the range of ChE activity in the plasma in non-exposed White stork.
- to document the influence of age in ChE activity in the plasma of the White stork.
- to investigate the *in vitro* sensitivity of these enzymes to OP and CBs. Paraoxon-methyl was used as model substance for OP, carbofuran and carbaryl were used as model substances for CBs.

2. Material and methods

2.1. Chemicals

Acetylthiocholine iodide, butyrylthiocholine iodide, propionylthiocholine iodide, iso-OMPA (tetraisopropyl pyrophosphoramide), eserine sulphate, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), BW284C51 (1,5-bis(4-allyldimethyammoniumphenyl) pentan-3-one dibromide), paraoxon-methyl (O,O-Dimethyl O-(4-nitrophenyl) phosphate; Purity: 98%), carbaryl (1-Naphthyl-*N*-methylcarbamate; Purity: 97%) and carbofuran (2,3-Dihydro-2,2-dimethyl-7-benzofuranol *N*-methylcarbamate; Purity: 98%) were purchased from Sigma-Aldrich Química SA, Portugal.

2.2. Animals

The study was performed on a total of 31 birds classified in two groups: adult ($n=13$) and juvenile ($n=18$) individuals that were being rehabilitated in the Los Hornos Wildlife Rehabilitation Centre in the Extremadura region, Spain, following physical injuries (e.g. electrocution or fall from the nest). Adults were more than 1-year old and juveniles were approximately 2 months old. They were allocated outdoor into a flight cage (20m × 20m × 5m), their food consisted of a diet based in cut up chicken, one day dead chick and water *ad libitum* throughout recovery period. The White stork is included in the List of Specially Protected Wild Species (Real Decreto 139/2011 de 4 de febrero para el desarrollo del Listado de Especies Silvestres en Régimen de Protección Especial y del Catálogo Español de Especies Amenazadas) in Spain. We were authorised by Consejería de Medio Ambiente (local government) to carry out the blood sampling.

2.3. Collection and preparations of samples

Blood sampling was conducted after the complete recovery of the animals which was achieved in a range of 15 days to 1 month depending on the case. The state of recovery of the birds was established by veterinarians of Wildlife Rehabilitation Centre on the basis of appropriate medical criteria (clinical symptoms and haematological and biochemical determinations). The birds were considered recovered when clinical symptoms had disappeared and the haematological (packed cell volume, haemoglobin, red blood cells and white blood cells) and biochemical (total proteins, glucose, aspartate amino-transferase AST–, creatine kinase –CK–, lactate dehydrogenase –LDH–) parameters measured were within the physiological range established for the species by different authors (adults: Szabó et al., 2010; and juveniles: Montesinos et al., 1997; Blázquez et al., 2006). Blood sampling was conducted after total recovery of the birds and immediately before their release in the wild. Blood was sampled in the morning, to avoid error due to circadian variations in enzyme activities or other blood parameters, and in the spring season. Whole blood was taken by puncture of the brachial vein using a syringe with heparin (25-gauge) and was mixed immediately in heparinized tubes (1.5 mg/dl). Plasma was obtained by centrifugation (2000*g*, for 5 min at 4 °C) and immediately stored at −20 °C until further analysis.

2.4. Catalytic properties

The substrates preferences of plasma ChE were investigated by determining the activity of ChE in the White stork plasma at increasing concentrations of acetylthiocoline (ASCh), butyrylthiocoline (BSCh) and propionylthiocoline (PSCh) (from 0.02 to 20.48 mM, incubation concentrations) in independent experiments, using Ellman's technique (Ellman et al., 1961) adapted to microplate following the general procedure indicated in Guilhermino et al. (1996). Briefly, the ChE activity