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Plasma cholinesterase activities in wild birds from undisturbed woodlands in the central Monte desert

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ABSTRACT

Plasma cholinesterase activity is a biomarker sensitive to the effect of organophosphate and carbamate pesticides. These enzymatic levels are unknown for most

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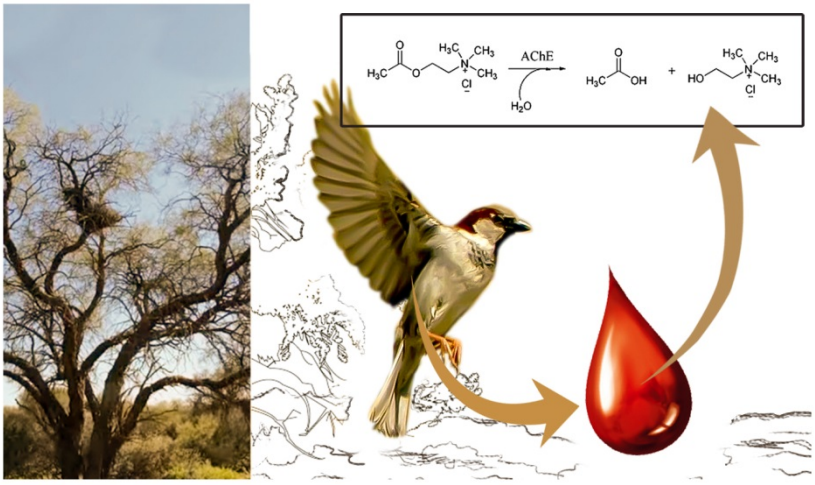
of the wild birds analyzed in this study. The objectives were to establish plasma acetylcholinesterase levels in songbirds of two undisturbed sites in the central Monte desert (Argentina). The influence of age, sex, body condition, feeding and migratory habits, and species of belonging on cholinesterase activity is also examined. One hundred and sixty-five wild birds belonging to 26 species were studied. The values obtained for acetylcholinesterase activities provide a good estimate of the normal values in free-living individuals of *Zonotrichia capensis*, *Molothrus bonariensis*, *Passer domesticus*, *Diuca diuca*, *Pospiza ornata*, *Saltator aurantiirostris*, *Gryseotyrannus aurantioatrocristatus* and *Columbina picui*, with interspecific differences. The media enzymatic levels ± standar error ranged from 546.31 ± 17.97 μmol min-1 L-1 in *P. domesticus* to 3439.90 ± 173.92 in *Tyrannus melancholicus*. No significant differences were detected between different sexes or ages. Birds that migrate (which are also insectivores), showed higher levels of cholinesterase than residents (mainly granivores). It is recommended that in cases of bird poisoning, plasma cholinesterase activity can be used as a diagnostic tool only if pre-exposure levels obtained in the same species are available, and ideally evaluated in individuals from the same biogeographical region.

Graphical abstract

Prosopis flexuosa, exponent of the central Monte desert; Passerines in study and plasma cholinesterase reaction.



Key words: plasmatic cholinesterase- wild birds- songbirds- variability factors

INTRODUCTION

Birds are one of the groups most sensitive to the toxic effects produced by organophosphate (OP) and carbamate (CB) anticholinesterase pesticides (Mitra et al. 2011; Ruvalcaba-Ortega et al. 2017). The LD50 of these compounds are between 10 to 20 times lower in birds than in mammals (Kaneko et al. 2008). Cholinesterase (ChE) enzyme catalyzes the hydrolysis of the neurotransmitter acetylcholine by inhibiting esterases in the central and peripheral nervous systems, ending the nerve impulse (Nunes 2011). Intoxication events with pesticides in humans and wild animals occur frequently (Guitart 2010; Sánchez Barbudo 2012). In our region, OP and CB constitute 49% of the total cases of poisoning in people living in agricultural areas (Toxicological Information Center, Mendoza), where aldicarb and carbofuran have been the causative agents of recent intoxications in canines and pigeons (Ferré et al. 2015; Saldeña et al. 2017). This

situation, added to the fact that are easily available, and the legal and illegal use of OP and CB, represents a great threat to birds (Oropesa et al. 2013). The inhibition of ChE in birds and mammals induces acute toxicity, with symptoms that include lack of coordination, weakness, ataxia, muscle tremor, diarrhea, seizures, breathing difficulties (respiratory distress), bradycardia, paralysis and fatal respiratory failure (Shimshoni et al. 2012). For the causal diagnosis of birds with signs of anticholinesterase intoxication in accidental or intentional events, it is necessary to have ChE activity levels in birds that reside in protected natural sites, apparently without exposure to pesticides. The inhibition of cerebral AChE has been used as a biomarker of neurotoxicity against OP and CB, where a 20% decrease in its activity has been diagnostic of exposure to this group of pesticides (Mineau 2002). To avoid the sacrifice of the animals, the plasmatic ChE activity is used, which is also a biochemical biomarker of exposure to OP and CB (Strum et al. 2010, Oropesa et al. 2013). In addition, plasma AChE activity is also implemented as a biomarker for environmental analysis (Nunes 2011), as a useful tool to evaluate in birds the possibility that some pesticides are polluting the environment (Stuber et al. 2018). Environmental factors that can produce variations in ChE activity have been reported (Tecles and Cerón 2001). ChE activity in birds is subject to interspecific and intraspecific species variations (Roy et al. 2005; Cobos et al. 2010). Therefore, it is important to determine the ChE activity reference intervals in each species (Horowitz et al. 2016).

The Monte desert occupies an extensive area in the west of Argentina. Despite being an arid region, it has a great ornithological diversity (Cueto et al. 2008), from protected areas with absence of agricultural activity and use of pesticides, to areas where

fruit and vegetables are cultivated where anticholinesterase insecticides are widely used (Ferré et al. 2018). The measurement of plasma AChE activity levels has not been previously performed in bird species representative of the Monte desert. The objective of the present study was to analyze the plasma AChE activity of several wild bird species that live in undisturbed natural sites of the Monte phytogeographic region, Argentina. We also analyzed, when possible, whether there is a relationship between the levels of AChE activity and factors such as species category, sex, age, feeding and migratory habits.

MATERIALS AND METHODS

The research was carried out in two protected sites of the central Monte desert (Mendoza, Argentina): The Telteca Forest Reserve and the Ñacuñán Biosphere Reserve, located 233 km apart from each other (Figure 1). These sites represent areas in which no agricultural activity is carried out and both provide a breeding habitat and/or migratory scale for more than 100 species of wild birds throughout the year (Cueto et al. 2008). The climate is arid with high evaporation rates, wide variations in temperature, from 16°C in winter up to 40-42ºC in summer, rainfall ranges from 200-400 mm per year, concentrated mainly in summer (Abraham et al. 2009). The characteristics of the vegetation in these sites are shrub steppes with a majority of *Larrea* spp. (Zygophyllaceae) and *Prosopis flexuosa*, which forms open forests (Villagra et al. 2009). Both have been identified as IBAs (Important Bird and Biodiversity Areas) (http://www.birdlife.org).

Sampling of birds

The birds were captured using mist-nets (12 m × 3 m, 34 mm). Nets were deployed from 6 am to 8 pm and were checked every 20 min. Each bird was removed

from the nets by a systematic procedure (Ralph et al. 1996). The birds were classified following Remsen et al. (2015). We performed an external examination estimating the visible fat depots in the interclavicular region (furcular fat) using a relative scale from 0 to 3 (Ralph et al. 1996). The birds showed no signs of intoxication. Sex in the species with external sexual dimorphism, and age (according to the presence of labial commissure) were classified as adults or juveniles.

The blood samples were obtained from the brachial vein, collected in heparinized capillary tubes, and kept at 4°C until processing. The plasmas were obtained by centrifugation at 10,000 rpm for 5 min. They were refrigerated and transferred to the Laboratory of Genetics, Environment and Reproduction at the University Juan Agustín Maza where they were kept at -80°C until their analysis. Sampling was conducted between October and December 2017, and the birds were released in the original place of their capture. The fieldwork protocol has been evaluated and approved by the Institutional Committee for the Care and Use of Laboratory Animals, Research and Teaching (CICUALID-UMaza) (Protocol No. 119).

Determination of cholinesterase activity

Validation of the method for determining AChE activity: The analysis was carried out according to the method of Ellman et al. (1961) modified by Trudeau and Sans Cartier (2000). The 5,5-dithiobis-2-nitrobenzoic acid (DTNB Aldrich®) (0.26 mmol / L) in IFI® phosphate buffer solution at pH 7.5 was used as the chromogen; and a solution of acetyl thiocholine iodide (CAS No. 1866-15-5 Sigma®) as a substrate (0.156 M). The determination was carried out at 25°C, at 405 nm in a spectrophotometer (Varian Cary

UV®) at 0, 30, 60 and 90 sec. For all samples, a single measurement was taken, due to the low volume of blood extracted from each specimen, which would constitute less than 1% of body weight (Fair et al. 2010).

The linearity of the enzymatic activity was tested with a plasma sample of *Molothrus bonariensis*, the passeriform with the highest volume of blood extracted, enough to perform the linearity curve in triplicate. The following concentrations were used: undiluted plasma (100%) and increasing dilutions with physiological solution: 50%, 25%, 12.50%, and 6.25% plasma. The coefficient of variation (CV) of each triplicate was obtained. The determinations in all the birds were made with the same batch of chromogen and substrate.

Statistic analysis

The linearity of the determination of AChE activity was evaluated by Pearson's linear regression test between the different dilutions of the blood plasma and the enzymatic activity. AChE activity levels are presented as mean ± standard error (SE), minimum and maximum values by species and by site under study. As a pre- stablished criteria, the possible effects of different factors on the enzymatic levels were analyzed only among those species in which $n \geq 5$ individuals were available. We analyzed whether there was a correlation between the variables sex, age, body weight, fat interclavicular depots, residence status (resident and migrant) and food guilds (omnivores, granivores, insectivores) of individuals with plasma AChE levels using the Spearman Test. To evaluate factors of variation effects, data were log normalized and a Mixed Generalized Linear Model (GLMM) was used, using the t-tests of Satterthwaite's

method with Imer 1.0 package (Kuznetsova et al., 2017) in R v. 3.3.2 (R Core Team 2016). The AChE activity was used as the dependent variable and the site as a random variable. InfoStat software version 2016 was used to design graphics.

RESULTS

In the AChE activity linearity study, the range of dilutions tested showed a high relationship between plasma dilutions and enzymatic activity: $r^2 = 0.98$, $p \leq 0.001$ (Figure 2). The average enzymatic activity (mean ± SE) of an initial undiluted plasma sample was 1832.15 ± 41.15 μmol min-1 L-1 (CV: 3.96%); and the diluted ones were: 1: 2 dilution with 722.67 ± 1.19 μmol min-1 L-1 (CV: 0.29%); 1: 4 dilution with 351.42 ± 0.59 μmol min-1 L-1 (0.29%); 1: 8 dilution with 212.20 ± 1.73 μmol min-1 L-1 (CV: 1.41); 1:16 dilution with 130.95 ± 1.24 μmol min-1 L-1 (CV: 1.64%).

In the study, a total of 165 wild birds belonging to 26 different species were captured from the 2 sites (Table 1). The birds studied looked healthy; body weight and fat depots were shown in supplemental data. The minimum and maximum AChE value varies among individuals of the same species generally around 2.5- fold, and extended from 1.2- fold in *Patagioenas maculosa* to 4.7-fold in *Poospiza ornata*. Six species were represented in both Reserves with similar intraspecific AChE activities. The species and their average levels of AChE were: *Zonotrichia capensis* 2086.99 ± 89.47 (n = 35), *M. bonariensis* 1691.31 ± 122.56 (n = 20), *Passer domesticus* 546.31 ± 17.97 (n = 10), *Saltator aurantiirostris* 1724.05 ± 142.38 (n = 11), *Turdus amaurochalinus* 1683.24 ± 627.51 (n = 2) and *Columbina picui* 943.65 ± 82.91 (n = 13).

The range of AChE activity between species was extended from *P. domesticus* with the minimum mean to *Tyrannus melancholicus* with the maximum one (6.2- fold). Then, the analysis model for the possible factors of variation was made in a total of 8 species according to the pre-established criteria. The variability among all them is shown in table 2.

The analysis between genders could be carried out in 3 species which presented clear external sexual dimorphism: *M. bonaerensis*, *P. ornata* and *S. aurantiirostris*. The AChE activity between females and males in those species that show sexual dimorphism had P = 0.1785 (df = 37.05, T = 1.371, Figure 3A). The analysis between ages could only be performed in *P. domesticus*, and the plasma activity between adults and juveniles had P = 0.5879 (df = 22.02, T = 0.55,140, Figure 3B). The correlation between AChE levels and body weight (supplemental data) by species could be evaluated in 8 species and negative correlation was observed only in *Z. capensis* (p = 0.01, r = -0.43). The correlation analysis between fat interclavicular depots (supplemental data) and levels of plasma AChE activity showed that there was no association between both variables for each any species analyzed. In the analysis between residence status, it was observed that for the total captures birds, 62.5% were resident species and 37.5% were migratory species. The plasma AChE activity between migrants and residents had P = 0.0007 (df = 127.00, t = 3.49, Figure 3C). The plasma AChE activity was P = 0.0045 (df = 1.00, T = 142.142) in granivores and P = 0.0005 in insectivores (df = 113.64, T = 3.602, Figure 3D) compare with omnivores.

DISCUSSION

Choosing a single ChE end point is required when only small volumes of blood samples may be collected in studies such as ours that sample passerines. This end point also allows a comparison across species. We selected the Ellman method, with acetylthiocholine as a substrate (González-Escalante et al. 2013; Stuber et al. 2018), due to the high specificity of the AChE (Miao et al. 2010) and the availability of data in the bibliography to make comparisons between species and sampling sites. On the other hand, characterizing and selecting the most appropriate substrate in each species, for acetyl, butyryl or propionyl ChEs, is the procedure indicated when the first step of biomonitoring is to identify a species for its use as a focal species (EFSA 2009). It is also appropriate to indicate that inter-laboratory variability in the determination of AChE has also been reported, even when using the same basic analytical method (Tecles and Cerón 2001). Given this reality, we believe that for biomonitoring situations, each laboratory should have data on its own reference control groups.

In the present study it is observed an overall variability in the range of AChE activity between species, higher than the inter-individual variatons inside each species. The high variation in AChE activity could potentially be due to intrinsic characteristics of the species. If we refer to other taxonomic classes, for example mammals, the ChE activity also shows differences between species (Kaneko 2008). Some authors postulate that phylogeny is one of the factors that may explain, in part, the variability in ChE activity (Roy et al. 2005), and that plasma ChE has been more influenced by the genotype of birds than by the environment (Norte et al. 2009). In the present study, the maximum value of AChE activity was detected in *T. melancholicus*, which exceeds by 9 times the

minimum value detected in *P. domesticus*, which exhibited the lowest value of ChE (0.546 ± 0.017 μmol-1min-1L, n = 10). *P. domesticus* is a species with a close relationship with humans, is an obligate commensal of sedentary humans (Anderson 2006). It has been observed in other bird species, that the groups that were living with humans had lower levels than the groups without daily contact (Swarg 2012). On the other hand, in the studies conducted by González-Escalante et al. (2013), *P. domesticus* seems to be the species most sensitive to the inhibition of ChE activity among the species studied. The average value that we have obtained of ChE activity for *P. domesticus* that inhabit the Monte desert has been lower than that reported in Mexico for non-agricultural area individuals: 1,379 ± 0.338 μmol-1 min-1L (n = 7) and agricultural area ones: 0.998 ± 0.289 μmol-1 min-1L, (n = 7).

We did not find differences between males and females, nor between juveniles and adults (Fig. 2 a and b) in the species that we were able to evaluate. Sex, age, physiological and reproductive status, have been indicated as causes of biological variability in human, rodent, and domestic animal plasma ChE activities (Tecles and Cerón 2001; Vaughan-Higgins 2016). In birds, it has been reported that different sexes and ages differ in their enzymatic biochemistry and physiological activity (Maul and Farris 2004; Roy 2005; Norte et al. 2009), but it is not a situation well defined since other studies like ours did not find any differences (Zwarg et al. 2012). This would be important to define since other authors indicate that juvenile birds may be more sensitive than adults to compounds that inhibit ChEs (Kaneko et al. 2008).

It has been reported that migrating birds may be particularly susceptible to exposure and negative effects of neurotoxic insecticides, because they may stray into

fumigated crops during their migrations (Smith and McWilliams 2014). However, in our study, the migrant species showed higher mean AChE activity values than the resident species which hypothetically are less likely to move from the large reserves sites to the agricultural sites. For at least 10km around the sampling site there is no land being used agriculturally or with apparent application of pesticides. We believe that the possible exposure to agricultural pesticides in resident birds can only be linked to the distances of displacement, where large ones would increase the chances of entering agricultural fields.

The majority of the individuals we captured were granivores and insectivores birds, in line with the main trophic groups reported for these sites (Cueto et al. 2008). The granivore species had the lowest enzymatic activity means, while the insectivore species showed the highest values. Globally, granivore birds can be seriously impacted by several insecticides (BLI 2018). Taking into consideration the food consumed by the species, could indicate the potential risk agents for birds. In agricultural areas, the possibilities of intoxication in birds are given by the type and rate of intake (EFSA 2009). Therefore, knowledge about diets would be valuable for the evaluation of possible exposure scenarios.

In conclusion, the substantive importance of the research lies in the fact that there were no available enzymatic data indicative of neurotoxicity for these birds species that inhabit this desert. In addition, in this same vast phytogeographic region there are other sites, where agricultural activities are carried out with neurotoxic pesticides, in which the same species of birds inhabit. If in these areas we want to evaluate the effect of fumigations, we should have reference values like those of the present study, ideally in the same species. The study is of clinical importance for veterinarians who treat cases of

intoxicated birds, and has scientific relevance because we have studied biochemical aspects not previously addressed in birds of this region. Plasma AchE enzymatic activity levels are proposed for the first time for 22 species of Passeriformes, 2 Columbiformes, 1 Psitaciformes and 1 Piciforme that inhabit the central Monte region. In the 8 species in which $n \geq 5$ individuals were obtained: *Z. capensis, M. bonariensis, P. domesticus, Diuca diuca, P. ornata, S. aurantiirostris, Gryseotyrannus aurantioatrocristatus* and *C. picui,* inter-specific differences in the AChE levels are shown. These can be considered pre-exposure levels for comparative studies in the future.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.xxxx.

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Data accessibility—Data, associated metadata, and calculation tools are available from the corresponding author (noragorla@gmail.com – aamartinquero@gmail.com).

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Figure 1. Map showing the Biogeographic province of the central Monte desert (shaded) in perspective from Argentina and South America. T: Telteca Natural Reserve (32°23′27″ S- 68°01′30″ W, 540 masl); Ñ: Ñacuñán Biosphere Reserve (34°03’0″ S - 67°58’0″ O, 583 masl).

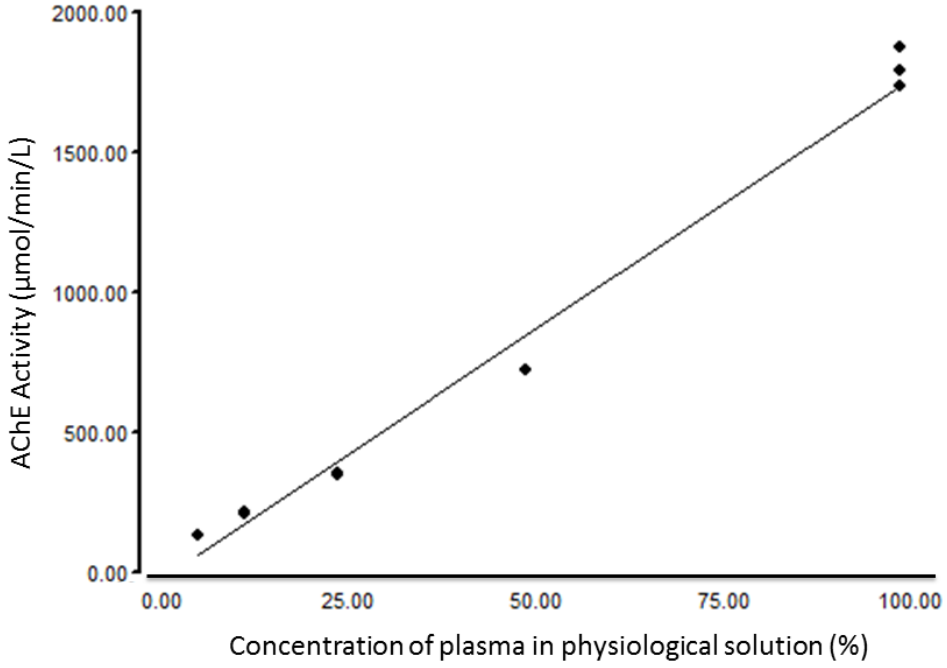
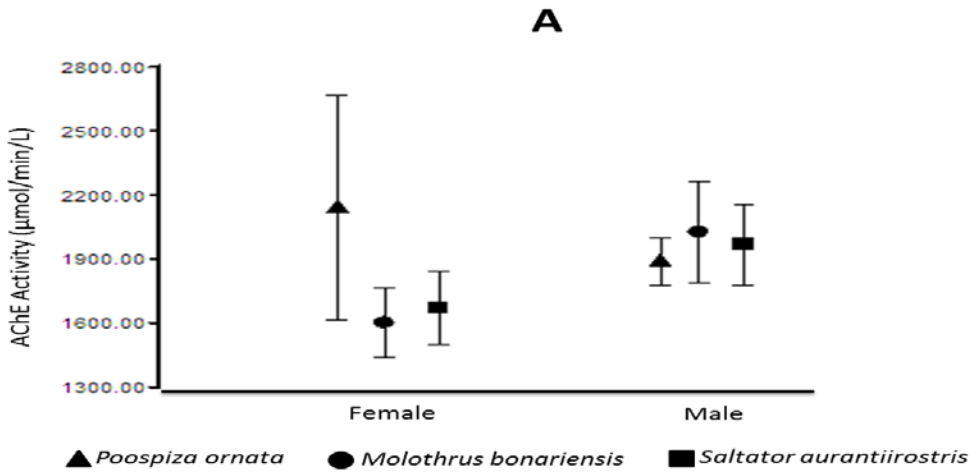
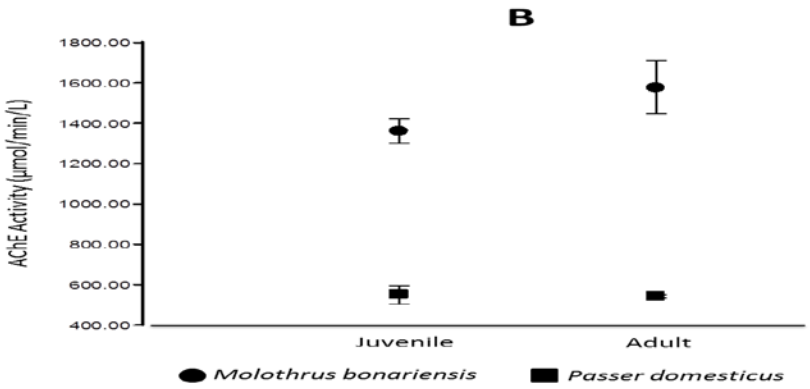
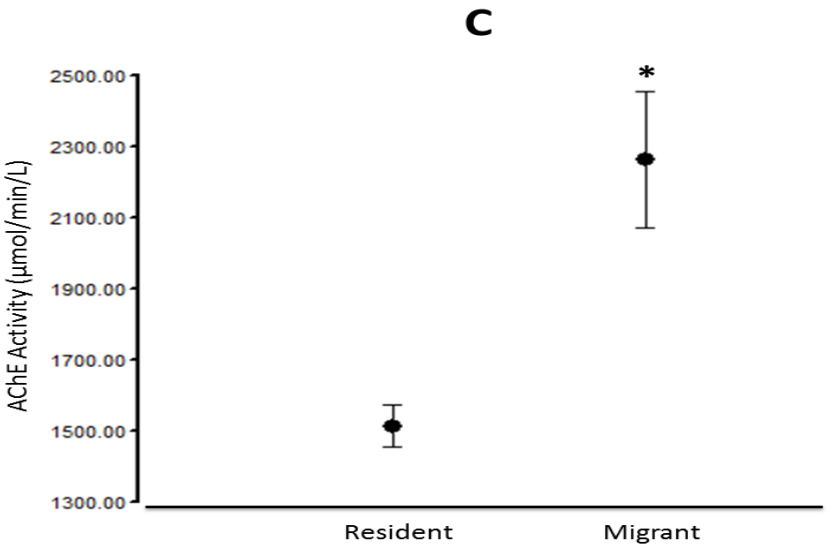


Figure 2. Linearity of the acetylcholinesterase activity in a wild Passerine plasma (r^2 = 0.98) p≤0.001. AChE: acetylcholinesterase. The determination was performed in plasma obtained from a *Molothrus bonariensis* individual without anticholinesterase chemical exposure.







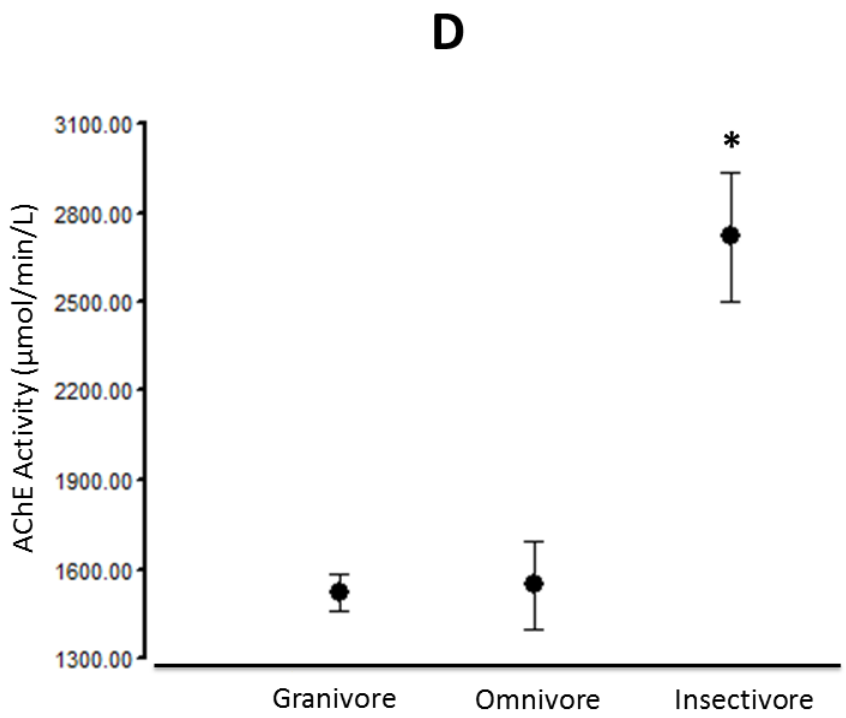


Figure 3. Analysis of plasma cholinesterase activity according to different factors in wild birds from central Monte desert. AChE: acetylcholinesterase ± standard error. **A:** Sex, *Molothrus bonariensis:* females (n = 6) and males (n = 11), *Poospiza ornata:* females (n= 4) and males (n= 4), *Saltator aurantiirostris:* females (n = 5) and males (n =6). **B:** Age, *M. bonariensis: juvenile* (n = 3) and adult (n = 12), *Passer domesticus:* juvenile (n = 4) and adult (n =6). **C:** Residence status, resident (n =123) and migrant (n= 26). *: $p < 0.001$. **D:** Feeding habits, granivores (n =117), insectivores (n = 18) and omnivores (n = 14). * $p < 0.001$.

Table 1. Plasma cholinesterase activity in wild birds from central Monte desert protected areas, Argentina.

				Ñacuñán Biosphere Reserve					Telteca Natural Reserve				
Species	Common Name	Mor Status	Fed-ing guild	n	Mean AChE activity *	SE	Min	Max	n	Mean AChE activity *	SE	Min	Max
Passeriformes													
Emberizidae													
Zonotrichia capensis	Rufous-collared sparrow	R	G	25	212 7.6	11 1.7	96	336	10	198 5.4	14 5.1	12 30.1	284 2.7

Furnariidae													
Furnarius rufus	Rufous hornero	R	I	-	-	-	-	-	2	257 8.5	91 2.8	16 65.7	349 1.3
Tarphonomus certhioides	Chaco earth-creeper	R	I	-	-	-	-	-	1	255 3.3	-	-	-
Icteridae													
Agelaioides badius	Bay-winged cowbird	R	G	1	284 7.0	-	-	-	-	-	-	-	-
Molothrus bonariensis	Shiny cowbird	R	G	15	153 4.6	10 7.4	10 55.3	22 99.0	5	216 1.3	30 1.1	10 40.5	271 4.7

	bird												
Mimidae													
Mimus saturninus	Chalk-bored mocking-bird	R	O	2	153 0.4	29 7.6	12 32.8	18 27.4	-	-	-	-	-
Mimus triurus	White-banded mocking-bird	M	O	3	890 .5	14 1.2	65 2.1	11 40.7	-	-	-	-	-
Paseridae													
Passer domesticus	House sparrow	R	G	7	552 .2	25 .3	44 3.4	65 4.4	3	532 .5	14.9	51 2.1	561 .6

Thraupidae													
Diuca diuca	Common diuca finch	R	G	-	-	-	-	-	24	120 3.4	56.9	75 9.3	192 1.53
Paroaria coronata	Red-crested cardinal		G	1	190 7.1	-	-	-	-	-	-	-	-
Phrygilus gayi	Grey-hooded sierra finch	R	G	-	-	-	-	-	3	170 1.3	39 4.9	92 4.7	221 4.0
Poospiza ornata	Cina	M	G	8	192	27	66	31	-	-	-	-	-

	mon war blin g-finc h				1.6	3.0	0.3	34.0						
Saltator aurantiirostris	Gol den-bille d salta tor	R	O	1	166 7.2	-	-	-	1 0	172 9.6	15 7.3	91 3.8	252 9.1	
Pipraidea bonariensis	Blue and Yell ow tana ger	R	G	-	-	-	-	-	1	251 7.4	-	-	-	
Turdidae														
Turdus amaurochalinus	Crea my-	R	G	1	231 0.7	-	-	-	1	105 5.3	-	-	-	

	belli				5								
	ed												
	thru												
	sh												
Tyrannidae													
Gryseotyrannus	Cro	M	I	8	265	24	19	37	-	-	-	-	-
aurantioatrocrista	wne				4.5	5.	91.	77.					
tus	d					1	3	9					
	slaty												
	flyc												
	atch												
	er												
Myiarchus	Swa	M	I	1	832	-	-	-	-	-	-	-	-
swainsoni	n-				.3								
	son’												
	s												
	flyc												
	atch												
	er												
Myiarchus	Bro	M	I	1	617	-	-	-	-	-	-	-	-
tyrannulus	wn-				.4								

	crested flycatcher												
Myiodynas-tes maculatus	Streaked flycatcher	M	I	2	216 0.0	45 8. 4	17 01. 6	26 18. 5	-	-	-	-	-
Tyrannus melancholicus	Tropical kingbird	M	I	4	343 9.9	17 3. 9	31 08. 7	39 30. 0	-	-	-	-	-
Tyrannus savanna	Fork-tailed flycatcher	M	I	3	192 5.2	61 4. 7	11 07. 2	31 28. 9	-	-	-	-	-

Xolmis coronatus	Black-crowned monjita	M	I	-	-	-	-	-	1	813.1	-	-	-
Columbiformes													
Columbidae													
Patagioenas maculosa	Spot-winged pigeon	R	G	3	1225.9	89.2	1047.5	1319.8	-	-	-	-	-
Columbina picui	Picui ground dove	R	G	8	877.9	92.9	494.1	1198.5	5	1048.8	158.03	726.18	1548.7

Psitaciformes														
Psitacidae														
Myiopsitta monachus	Monk parakeet	R	G	-	-	-	-	-	4	122 2.5	22 4.9	83 2.3	165 9.8
Piciformes														
Picidae														
Melanerpes cactorum	White-fronted woodpecker	R	I	-	-	-	-	-	1	531 8.8	-	-	-
Total individuals				9 4					7 1					

M: migrant. R: resident. AChE: acetylcholinesterase. *µmol/min/L. SE: standard error. Min: minimum. Max: maximum. G: granivores. O: omnivores. I: insectivores.

Table 2. Results of Generalized Linear Model showing variability in acetyl cholinesterase activity among 8 species of wild birds from Monte desert preservation areas. Degree of freedom, T value and p value are showed.

Species*	Common name	d.f.	t value	p
Zonotrichia capensis	Rufous-collared sparrow	120.81663	8.727	1.73 e-14*
Molothrus bonariensis	Shiny cowbird	120.98604	5.728	7.56 e-08*
Passer domesticus	House sparrow	120.43590	-4.191	5.33 e-05*
Diuca diuca	Common diuca finch	31.97476	2.610	0.0137*
Pospiza ornata	Cinamon warbling-finch	109.75228	5.263	7.10 e-07*
Saltator aurantiirostris	Golden-billed saltator	72.15671	5.178	1.95 e-06*
Gryseotyrannus aurantioatrocristatus	Crowned slaty flycatcher	109.75228	8.223	4.41 e-13*
Columbina picui	Picui ground dove	18.32000	84.694	< 2 e-16*

* The inter-specific differences were analyzed among those species in which n ≥ 5 individuals were available.