

# Brain cholinesterase reactivation as a marker of exposure to anticholinesterase pesticides: a case study in a population of yellow-legged gull *Larus michahellis* (Naumann, 1840) along the northern coast of Portugal

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**Abstract** Between late 2010 to early 2011, an increased mortality in gulls was observed along the northern coast of Portugal, with individuals exhibiting neurologic disorders consistent with an eventual anticholinesterase pesticide poisoning event. To clarify if this mortality was related to organophosphate (OP) and/or carbamate (CB) poisoning, chemical and spontaneous cholinesterase (ChE) reactivation was tested in the brain of the yellow-legged gull (*Larus michahellis*). Initial brain ChE activity in *L. michahellis* was  $40.92 \pm 5.23$  U/mg of protein (average  $\pm$  SE). Following chemical and spontaneous reactivation, ChE activity increased in average  $70.38 \pm 48.59$  % and  $131.95 \pm 92.64$  %, respectively. ChE reactivation was found to decrease at increasing concentrations of the oxime pyridine-2-aldoxime methochloride and dilution factor, underscoring the importance of first optimizing the assay conditions prior to its use on bird species. These results suggest that birds analysed could have been exposed to OP and CB pesticide compounds and that in most cases CB exposure appeared to be the main cause of birds poisoning. These results are an important contribution to environmental monitoring as it demonstrates the suitability of *L. michahellis* as

sentinel species of OP and CB pesticides within an urban environment.

**Keywords** Avian toxicity · Biomarkers · Carbamate pesticides · Chemical reactivation · Organophosphate pesticides · Spontaneous reactivation

## Introduction

Present in the nervous system of both vertebrates and invertebrates, cholinesterases (ChE) are a family of enzymes that catalyse the hydrolysis of the neurotransmitter acetylcholine into choline and acetic acid (Frasco et al. 2005; Voet and Voet 1990). Nerve impulses pass across synapses through release of acetylcholine which, after the stimulating signals are transmitted, is readily hydrolysed to allow other signals to pass. Acetylcholinesterase (AChE; EC 3.1.1.7), which serves to terminate synaptic transmission, belongs to this family of enzymes and is mostly found in the central nervous system and neuromuscular junctions of vertebrates (Radic and Taylor 2006). In several avian species, AChE has also been reported to be the predominant form present in the brain (Osten et al. 2005).

ChE activity is particularly prone to inhibition by carbamate (CB) and organophosphate (OP) pesticides. This, in return, is known to cause an over accumulation of acetylcholine in the synaptic cleavage, leading to nerve overstimulation which in turn may trigger a wide range of adverse effects, from convulsions to paralysis and, ultimately, death (Frasco et al. 2005; Manahan 2003).

Amongst other vertebrates, birds are particularly vulnerable to OP and CB exposure, which may occur through ingestion of contaminated water, food, dermal contact and

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inhalation (Fleischli et al. 2004; Hill 2003). Avian poisoning by OP and CB has been extensively documented in the literature, from small Passeriformes such as the house sparrow (*Passer domesticus*) or the goldfinches (*Carduelis carduelis*) to medium-large water bird species such as gulls (*Larus* spp.) and shags (*Phalacrocorax aristotelis*) (Guitart et al. 2010).

Over the years, several studies have successfully used the inhibition of ChE activity as a tool to diagnose OP and CB exposure. Nonetheless, this approach has some limitations. For instance, the results obtained might be difficult to link with adverse effects at the organism level and/or to compare with basal levels of ChE activity, which are often not available or cannot be obtained for the studied species (Escartín E, Porte C 1996). Additionally, ChE activity may also be inhibited by other environmental contaminants such as petroleum-derived products, metals and detergents (Frasco et al. 2005; Guilhermino et al. 1998; Oropesa et al. 2007), which, even though ChE activity is comparatively more sensitive to OP and CB exposure, may act as a confounding factor when diagnosing pesticide poisoning. The use of ChE reactivation techniques has been suggested in the literature as an alternative to demonstrate acute ChE inhibition triggered by OP and CB pesticide poisoning (Escartín E, Porte C 1996; Stansley 1993).

Inhibition of brain and plasma ChEs by OP and CB pesticides originates phosphorylated and carbamoylated enzyme intermediates, respectively, which can be reversed by using simple in vitro procedures (Stansley 1993; Trudeau and Cartier 2000). Phosphorylated ChEs may have their activity restored chemically by nucleophilic reagents such as the oxime pyridine-2-aldoxime methiodide (2-PAM) (Martin et al. 1981; Thompson 1999; Trudeau and Cartier 2000). Briefly, the partially electropositive nitrogen of 2-PAM is attracted to the anionic site of the ChE, which enables 2-PAM to bind to the site where the OP inhibitor was previously attached and then bind to the electronegative phosphorus atom. This allows the removal of the phosphorus atom from the enzyme, enabling ChE reactivation (Fig. 1). The specificity of 2-PAM nucleophilic properties toward OP compounds has been commonly accepted (Goldstein et al. 1999; Hooper et al. 1989; Stansley 1993). Nonetheless, the effectiveness of chemical reactivation by 2-PAM and other nucleophilic agents is reduced by the dealkylation (aging) of the phosphorylated enzyme (Trudeau and Cartier 2000).

Carboxylated ChEs are generally less stable and therefore more prone to spontaneous hydrolysis than phosphorylated ChEs. Reactivation of a carboxylated enzyme can be promoted by thermal incubation, sample dilution, which minimizes the effects of excess inhibitor, or by removing inhibitor through dialysis or gel filtration (Manahan 2003; Smith et al. 1995; Stansley 1993). Contrarily to OP-inhibited ChEs, CB-inhibited ChEs are not reactivated by 2-PAM (Martin et al. 1981; Stansley 1993). These differences have been used as

baseline to distinguish between OP and CB exposure (Martin et al. 1981; Trudeau and Cartier 2000).

The main objective of this study was to investigate if an increased mortality of yellow-legged gulls (*Larus michahellis*) observed between late 2010 and early 2011 along the northern coast of Portugal was related to OP and/or CB poisoning. In order to clarify this hypothesis, chemical and spontaneous ChE reactivation was evaluated in the brain tissue of yellow-legged gull individuals collected from that mortality event.

## Material and methods

### Collection and preparation of samples

The birds submitted for analysis ( $n=11$ ) arrived alive to the veterinary centre of Gaia's Biological Park, a nature reserve located in Avintes (Porto, Portugal) with the symptoms of diarrhea and neurologic dysfunctions, including muscular weakness and ataxia, which are consistent with anticholinesterase pesticide poisoning. After death, their heads were removed, frozen immediately, and then transferred to the lab where they were kept at  $-80$  °C until tissue dissection and analysis.

Brain samples used to measure ChE activity and reactivation were homogenized (Yastral GmbH d-7801 Dottingen) in phosphate buffer (0.1 M, pH 7.2). Samples were kept on ice during all the procedure. All enzymatic and reactivation assays were performed on the supernatant obtained by centrifugation (3 min, 6000 rpm, 4 °C) of the initial homogenates.

### Chemicals

Acetylthiocholine iodide, 5,5'-Dithiobis(2-nitrobenzoic acid), bovine  $\gamma$ -globulin and 2-PAM were obtained from Sigma-Aldrich Europe (Netherlands). All the other chemicals used were purchased from Merck (Germany).

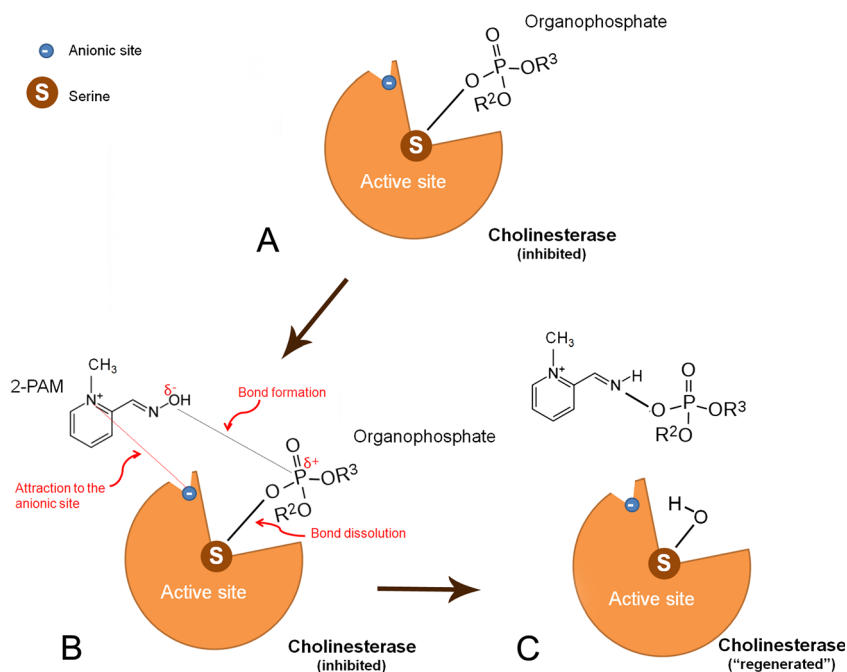
### Protein quantification

The concentration of protein in all samples was determined in quadruplicate by the Bradford method (Bradford 1976) adapted to microplate using bovine  $\gamma$ -globulin at 595 nm. Measurements were made using a Thermo Scientific Multiskan<sup>®</sup> Microplate reader from Spectrum.

### ChE determinations

ChE activity was determined at room temperature (25 °C) in quadruplicate, according to the Ellman method (Ellman et al. 1961) adapted to microplate (Guilhermino et al. 1996), using a microplate reader (Thermo Scientific Multiskan<sup>®</sup> Spectrum).

**Fig. 1** Chemical reactivation of a phosphorylated ChE with 2-PAM. **a** OP-inhibited enzyme, **b** 2-PAM binds to OP and its negatively charged atom of oxygen binds to the positively charged phosphorus atom of the OP, **c** The bond between the phosphorus atom and the oxygen of the serine is broken, and the OP molecule is removed from the ChE, restoring the enzyme normal function



To 50  $\mu$ l of sample (initial and after reactivation assays), 250  $\mu$ l of reaction mixture was added, and the absorbance at 414 nm was measured at 10, 15 and 20 min. Reaction mixture was prepared by mixing 30 ml of phosphate buffer (0.1 M, pH 7.2) with 0.2 ml of acetylthiocholine iodide (0.075 M) and 1 ml of DTNB (10 mM). In all ChE determinations, a blank analysis made of ultrapure water and the reaction mixture was also assayed to account for reagents absorbance. The enzymatic activity was expressed in units (U) per mg of protein (1 U is a micromole of substrate hydrolysed per minute).

### Reactivation assays

Reactivation of ChE was tested following an adaptation of the methods described by Martin et al. (1981). For the chemical reactivation assay, and for the supernatant of each brain homogenate, one aliquot of 250  $\mu$ l and seven aliquots of 200  $\mu$ l were separated. The 250- $\mu$ l aliquot was maintained on ice until assayed for initial ChE activity, and the others were used for the reactivation assay. Six aliquots were incubated with 200  $\mu$ l of 2-PAM (concentrations ranging from 0.01 to 5 mM) and one with 200  $\mu$ l of ultrapure water (water control), for 1 h at 25  $^{\circ}$ C. ChE activity was then measured as described above. To account for the differences of absorbance produced by 2-PAM during the enzymatic assay, an additional blank composed of 200  $\mu$ l of ultrapure water and 200  $\mu$ l 2-PAM was prepared for each concentration of 2-PAM used and incubated in the conditions above mentioned.

In order to study spontaneous reactivation, each brain homogenate was separated into two additional 250  $\mu$ l sub-samples. The first aliquot was kept on ice and the second aliquots

were successively diluted 10, 20, 40, 80 and 100 times with cold phosphate buffer (0.1 M, pH 7.2) up to a final volume of 500  $\mu$ l. Diluted samples and the undiluted aliquots were then incubated at 4  $^{\circ}$ C for 24 h, and ChE activity was determined as described above.

### Set up, data treatment and analysis

Enzymatic activity was determined for each sample before (i.e. initial activity) and after the reactivation assays. In samples assayed for chemical reactivation, absorbance of samples during the enzymatic reaction was corrected by subtracting the absorbance obtained in the additional blanks prepared with 2-PAM. The absorbance of all other samples was corrected using the blank of ultrapure water.

Percentage of reactivation (referred to hereafter and in the abstract) was calculated by converting the activity obtained in the reactivation assays into percentages, using the following formula:

$$\% \text{ of reactivation} = \left[ \left( \text{ChE}_R \times 100 \right) / \text{ChE}_I \right] - 100$$

$\text{ChE}_R$  = ChE activity after reactivation

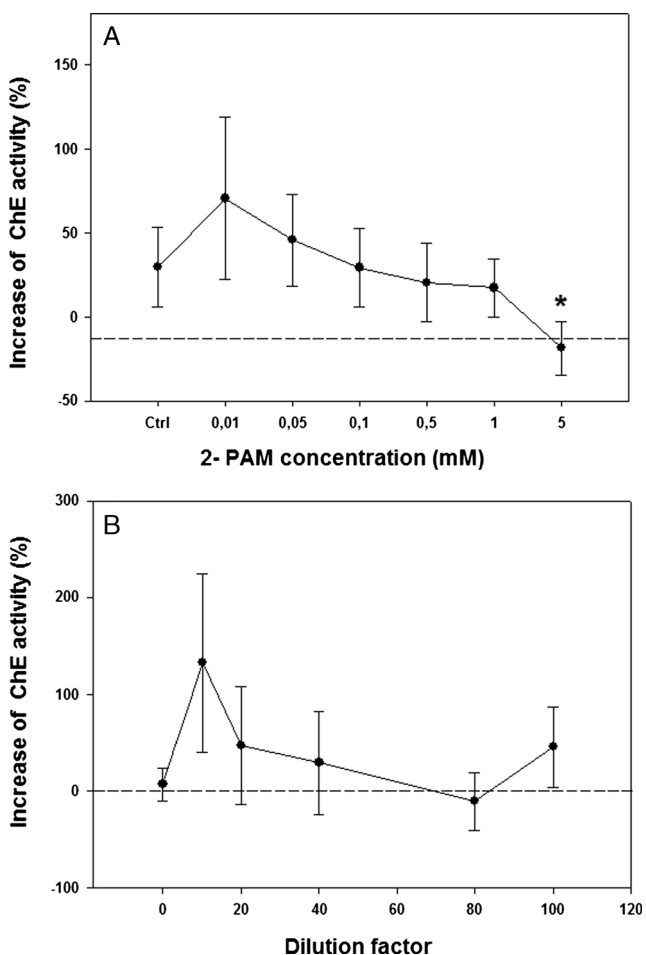
$\text{ChE}_I$  = Initial ChE activity

After conversion of ChE activity data to percentage of reactivation, reactivation curves were obtained later by removal of data outliers. Selection of outliers was done considering the mean  $\pm 2 \times$  standard deviation. Normality of variables was then checked using the Shapiro-Wilk Test. Data were found to deviate significantly from normality, and since data

normalization was not possible for all data sets, all statistical analysis performed were non-parametric. A Kruskal-Wallis analysis of variance (ANOVA on Ranks) was performed to compare differences between 2-PAM concentrations (chemical reactivation) and dilution factors (DFs, spontaneous reactivation). All data analyses were performed using SigmaPlot® 11 software (Systat Software Inc.).

### Results

In order to investigate ChE reactivation in the brain of *L. michahellis*, we assayed ChE activity before and after chemical and spontaneous reactivation using increasing concentrations of the oxime 2-PAM (chemical reactivation) or by successive dilutions of brain samples (spontaneous reactivation) (Fig. 2). Maximum enzyme reactivation in the chemical and spontaneous assays was obtained at 0.01 mM of 2-PAM (70.38±48.59 %) and after a factor dilution of 10 (131.95±92.64 %), respectively.



**Fig. 2** Performance of chemical (a) and spontaneous (b) reactivation assays in brain ChE of *Larus michahellis*. Results are expressed as the mean±SE of ten birds, Ctrl=ultrapure water control, asterisk symbol indicates significantly different from control (Dunn’s test,  $P<0.05$ )

Percentage of ChE reactivation was observed to decrease with increasing concentrations of 2-PAM, being significant at 5 mM of 2-PAM ( $P<0.05$ ). Percentage of ChE reactivation was also observed to decrease, although at no significant extent, with increasing DF, except at the highest DF (100) in which percentage of reactivation increased. Minimum reactivation percentages were registered in the chemical assay at 5 mM of 2-PAM ( $-18.69\pm15.82\%$ ) and in the spontaneous assay after applying a DF of 80 ( $-11.45\pm30.31\%$ ).

Brain initial and reactivated ChE activities are depicted on Table 1. Average initial brain activity in *L. michahellis* was  $40.92\pm5.23$  U/mg of protein. Regarding the chemical reactivation assay, generally no effect on brain ChE activity was observed when comparing the ultrapure water control (average activity,  $55.32\pm5.85$  U/mg of protein) with the initial brain ChE activity ( $P>0.05$ ). This was true for all individuals analysed, except for birds 1, 7 and 9 in which an increase of ChE activity was observed after incubating the samples with the ultrapure water control for 1 h at 25 °C.

After sample incubation with 0.01 mM 2-PAM, in most of the individuals analysed, brain ChE activity was also found to increase (not significantly) when comparing to initial ChE activity ( $63.74\pm9.83$  U/mg of protein). Maximum percentage of reactivation following the incubation of samples was about 290, 410 and 667 % for birds 1, 10 and 7, respectively. A low percentage of reactivation in the chemical assay was, though, recorded for bird 6. No ChE reactivation was observed in this assay for birds 3, 8 and 11.

Brain ChE did not vary greatly when incubating the samples for 24 h without dilution (average activity,  $39.46\pm3.48$  U/mg of protein). After dilution, however, brain ChE was found to increase steadily (average activity,  $99.25\pm39.71$  U/mg of protein), with increases in some cases of about 423.7, 867.0 and 869.9 % for birds 1, 7 and 9, respectively. Lowest percentages of reactivation following dilution were 0 % (birds no. 6, 8 and 10) and 5.3 % (bird no. 11).

### Discussion

The use of ChE activity as a biomarker to diagnose exposure of organisms to anticholinesterase compounds such as OP and CB pesticides has been routinely described in previous works (Fildes et al. 2006; Hooper et al. 1989; Hunt et al. 1995). This approach usually requires reference or basal activity values from healthy animals to infer ChE inhibition which in some cases is not available; for this reason, ChE reactivation techniques have been proposed as an alternative to identify OP and CB exposure (Escartin E, Porte C 1996; Stansley 1993).

The methodologies used in this work were based on the original work described by Martin et al. (1981). Enzyme kinetics are known to vary significantly amongst species and tissues (Thompson 1999); therefore in order to determine best

**Table 1** Brain ChE activity of *Larus michahellis* before (initial activity) and after reactivation assays<sup>a, b</sup>

Bird Record (no.)	Initial activity	Chemical reactivation		Spontaneous reactivation	
		Water control <sup>c</sup>	0.01MM 2-PAM (%)	Control after 24 h <sup>d</sup>	1:10 (%)
1	24.48	82.51	95.50 (290)	34.69	128.18 (423.7)
2	44.48	48.40	52.85 (18.8)	46.74	56.63 (27.4)
3	52.11	50.69	46.15 (0)	46.53	63.31 (21.5)
4	44.29	52.54	57.81 (30.5)	43.55	62.03 (40.0)
5	37.51	41.06	41.18 (9.79)	42.34	54.59 (45.5)
6	42.21	43.36	45.33 (7.4)	34.50	39.99 (0.0)
7	10.61	82.97	81.35 (667)	17.68	102.57 (867.0)
8	77.59	71.67	73.31 (0)	44.25	49.41 (0.0)
9	50.00	72.22	68.38 (36.8)	32.31	484.61 (869.9)
10	25.92	22.78	132.17 (410)	63.13	7.36 (0.0)
11	40.91	40.31	7.11 (0)	28.38	43.08 (5.3)
Average activity	40.92±5.23	55.32±5.85	63.74±9.83	39.46±3.48	99.25±39.71

<sup>a</sup> Activities expressed as U/mg of protein and values in brackets correspond to the percentage of reactivation

<sup>b</sup> Averages of activities are presented by mean±SE

<sup>c</sup> Enzymatic activity obtained when incubating the samples with ultrapure water (1 h, 25 °C)

<sup>d</sup> Enzymatic activity obtained after incubating the original (not diluted) samples for 24 h, 4 °C

assay conditions for chemical and spontaneous assays brain ChE reactivation was tested in *L. michahellis* under different concentrations of 2-PAM and sample dilutions, respectively. In this study, the enzymatic activity measured in brain showed an increase with lower concentrations of 2-PAM, but with no statistical significance. This is possibly a consequence of the high variation registered in the enzymatic activities, which could be due to dissimilar background field conditions. Namely, gulls studied might have been exposed to other types of anticholinesterase contaminants (e.g. metals, detergents and/or other classes of pesticides) which could have triggered cholinesterase inhibition or even promote the denaturation of the enzyme, altering the efficiency of the reactivation assays. Furthermore, other environmental stressors (biotic and abiotic), might have as well induced additional stress on the individuals.

An absence of significance was also found for all 2-PAM concentrations, except for 5 mM where the percentage of enzyme reactivation was reported to be significantly lower than the control, which is indicative of enzyme inhibition. Such inhibitory effect of 2-PAM has already been reported on fish and crab's AChE activities at the concentration of 0.1 mM (Monserrat and Bianchini 2000; Thompson et al. 1991). Testing chemical reactivation with 2-PAM at different concentrations allows to optimize the assay conditions and increase the efficiency of the chemical reactivation. Even though in this particular case no consistent statistical data was obtained, higher enzymatic recovery was observed when using 0.01 mM of 2-PAM. This concentration seems to be, therefore, the most appropriate for use in future studies with *L. michahellis*.

Optimization of spontaneous ChE reactivation was assayed by applying different DFs (1:10 to 1:100) to the brain samples analysed. Lowest performance of spontaneous reactivation was obtained after applying a DF of 80, with percentage of reactivation being negative ( $-11.45\pm 30.31\%$ ), meaning that in average cholinesterase activity decreased. By promoting sample dilution in the spontaneous assay, the concentration of enzyme in the sample is reduced. If the DF is too high, enzyme concentration in the sample might be too low to allow proper kinetics of the enzyme and diminish detection of photometric changes over time, thus reducing the assay efficiency and overly contributing to retrieve low values of activity.

Best performance of spontaneous reactivation was achieved at lower DFs (1:10) and, similarly to the chemical assay, no statistical differences between the different DFs tested and the assay control (DF=0) were reported. As abovementioned, this might have been a result of the high variability observed in the enzymatic activities as a result of the dissimilar environmental conditions to which birds may have been exposed in the field. Despite the absence of statistical significance to infer optimal DF to use in future studies, higher percentage of ChE reactivation was observed when diluting the brain samples in a DF of 1:10. Therefore, this DF is suggested as the most appropriate for the determination of brain ChE in *L. michahellis*. Both chemical and spontaneous reactivation assay conditions were optimized using a limited number of individuals. Therefore, even if in this study these conditions were the ones to which higher reactivation was observed, should be merely used as reference conditions and further confirmed in future works with this species.

Generally, average initial brain activity in *L. michahellis* (40.92±5.23 U/mg of protein) was found to increase following chemical (55.3±25.85 U/mg of protein) and spontaneous reactivation (99.26±39.71 U/mg of protein) assay, with the percentage of enzyme activity being restored up to 30 and 50 %, respectively (Table 1). In literature, it has been usually accepted that brain ChE activity depressions of about 20 % in birds, fish and invertebrates were indicative of exposure to anti-ChE compounds, while depressions greater than 50 % in dead animals have been considered as diagnostic of death due to ChE inhibition (Escartín E, Porte C 1996; Goldstein et al. 1999; Ludke et al. 1975). Complete reactivation of all inhibited enzyme is often difficult to conclude. Amongst other factors that could act as confounding factors (e.g. aging of phosphorylated enzyme, presence of other type of ChE-inhibiting substances, such as metals and/or detergents, and denaturation of the enzyme) (Maxwell et al. 2013), incubation conditions influence widely the performance of ChE reactivation, and thus, it is likely that even optimized conditions may not lead to complete reactivation of all inhibited enzyme. Fildes et al. (2006) have suggested a formula<sup>(A)</sup> to account for percentage of inhibition, which considering the aspects abovementioned, would represent a minimum level of inhibition. Applying that same formula<sup>(A)</sup> for each individual analysed (percentage of reactivation used to make the conversion was always the highest value of both assays), percentage of inhibition was observed to vary between 17.7 and 89.7 %. It seems, therefore, reasonable to suggest that the birds analysed were exposed to both OP and CB pesticides, up to concentrations high enough to induce impaired neurologic physiology, thus explaining the symptoms observed in these individuals.

$$(A) \%inhibition = \left( \frac{\% \text{ reactivation}}{100 - \% \text{ reactivation}} \right)$$

Gulls, including *L. michahellis*, are known to exhibit a very opportunistic scavenging behaviour, recurring often to human garbage dumps or agricultural lands to feed, prompting their exposure to OP and CB compounds. These observations were true for the majority of the birds studied except for gulls no. 6, 8 and 11 which showed both spontaneous and chemical brain ChE reactivations never higher than 10 %. This lack of reactivation could be related to the aging of OP-inhibited ChEs, as this process is known to inhibit irreversibly enzymes, even in the presence of 2-PAM (Martin et al. 1981; Trudeau and Cartier 2000) or to the previous exposure of individuals to other classes of ChE-inhibiting compounds (e.g. metals, detergents) (Frasco et al. 2005; Guilhermino et al. 1998).

Moreover, analysis of reactivation data for both chemical and spontaneous assays depicted in Table 1 allowed us to discriminate individual differences in the performance of both assays amongst animals analysed. For example, while on gulls

no. 1, 7 and 9, brain ChE activity was mainly reactivated spontaneously, with percentage of reactivation varying from 423.7 to 869.9 %, brain ChE of gull no. 10 was found to reactivate more readily after incubation with 2-PAM (percentage of reactivation=410 %). This might be indicative of a differential contribution of OP and CB exposure to the acute effects observed on the studied gulls, with CB exposure being most likely the main cause of poisoning as, in seven out of the eleven individuals analysed, the percentage of reactivation obtained spontaneously was markedly higher than with 2-PAM.

## Conclusions

Following brain ChE reactivation under different assay conditions, we observed variability in success of ChE activity restoration underscoring the importance of firstly optimizing chemical and spontaneous assay conditions prior to the use of these assays as markers of OP and CB exposure in birds. In this study, we were able to successfully reactivate brain ChE of *L. michahellis* up to 20 and 50 %, which has been accepted in the literature as indicative of exposure to ChE-inhibiting compounds and acute toxicity, respectively. Additionally, using both spontaneous and chemical assays allowed us to distinguish between OP and CB inhibition, enabling us to infer that CB exposure may have been the principal cause of death of the birds analysed.

This study addresses for the first time the use of brain reactivation techniques in a long-lived seabird species well adapted to human presence, in particular to urban environment, to infer exposure to OP and CB pesticides. This data demonstrates the suitability of *L. michahellis* as sentinel species of these chemicals within an urban environment.

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