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Environmental Pollution 132 (2004) 479–488
ENVIRONMENTAL POLLUTION
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Inhibition of plasma butyrylcholinesterase activity in the lizard *Gallotia galloti palmae* by pesticides: a field study

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Received 25 January 2004; accepted 3 May 2004

"Capsule": *Chemical reactivation of lizard BChE activity is a suitable diagnostic method for evaluating field exposure to organophosphorus and carbamate pesticides.*

Abstract

A field study was performed to evaluate the effect of exposure to organophosphorus (OP) and carbamate (CB) pesticides on the lizard *Gallotia galloti palmae*. Butyrylcholinesterase (BChE) activity was measured in the plasma of 420 lizards collected from agricultural and reference areas on the Island of La Palma (Canary Islands, Spain) in two sampling periods. Exposure to cholinesterase-inhibiting pesticides was evaluated by a statistical criterion based on a threshold value (two standard deviations below the mean enzyme activity) calculated for the reference group, and a chemical criterion based on the in vitro reactivation of BChE activity using pyridine-2-aldoxime methochloride (2-PAM) or after water dilution of the sample. Mean (±SD) BChE activity for lizards from agricultural areas was significantly lower (Fuencaliente site = 2.00 ± 0.98 μmol min^{-1} ml^{-1}, Tazacorte site = 2.88 ± 1.08) than that for lizards from the reference areas (Los Llanos site = 3.06 ± 1.17 μmol min^{-1} ml^{-1}, Tigalate site = 3.96 ± 1.62). According to the statistical criterion, the number of lizards with BChE depressed was higher at Fuencaliente (22% of males and 25.4% of females) than that sampled at Tazacorte (7.8% of males and 6.2% of females). According to the chemical criterion, Fuencaliente also yielded a higher number of individuals (112 males and 47 females) with BChE activity inhibited by both OP and CB pesticides. CBs appeared to be the pesticides most responsible for BChE inhibition because most of the samples showed reactivation of BChE activity after water treatment (63.3% from Fuencaliente and 29% from Tazacorte). We concluded that the use of reactivation techniques on plasma BChE activity is a better and more accurate method for assessing field exposure to OP/CB pesticides in this lizard species than making direct comparisons of enzyme activity levels between sampling areas.

Keywords: Butyrylcholinesterase; Lizard; Pesticides; Pyridine-2-aldoxime methochloride; Field exposure

1. Introduction

Butyrylcholinesterases (BChE, EC 3.1.1.8) are enzymes belonging to a group of hydrolases classified by Aldridge (1953) as B-type esterases. They are inhibited by organophosphorus (OP) and carbamate (CB) pesticides. As a result, along with acetylcholinesterase (AChE, EC 3.1.1.7), BChE inhibition has been used as an indicator of exposure in biomonitoring programs of pesticide contamination. BChEs are found in the blood of many vertebrate species (Thompson and Walker, 1994), and although their physiological role remains uncertain, they appear to have a protective function by sequestering circulating OP compounds, thereby decreasing the toxic effect of these compounds on brain AChE (Russell and Overstreet, 1987; Allon et al., 1998); inhibition of AChE

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doi:10.1016/j.envpol.2004.05.008

is the primary mechanism of acute toxicity for OP and CB pesticides. The non-lethal nature of blood BChE measurement makes it a desirable biomarker when endangered or protected species are used as bioindicators. However, the use of this biomarker in field monitoring programs of pesticide pollution has been questioned. The rapid recovery of blood BChE activity after inhibition by OPs or CBs (Sanchez-Hernandez, 2001) and its high inter-individual variability (Walker, 1998) may result in an inability to detect BChE inhibition following agricultural pesticide applications.

There is a substantial body of literature describing the effects of exposure and toxicity of OP and CB pesticides on aquatic organisms (Fulton and Key, 2001), birds (Hill, 2003) and mammals (Sheffield et al., 2001). However, two reviews have recently stressed the need for increasing the understanding of the adverse effects of environmental contaminants on lizards and other reptiles (Campbell and Campbell, 2000, 2002). In addition, Pauli and Money (2000) have examined the available information concerning the toxic effects on reptiles of the main pesticide groups. They point out that very few data exist concerning the effects on reptiles of field pesticide applications. In this sense, our research group has investigated the use of measurements of BChE and AChE activities in the lizard *Gallotia galloti* and has suggested that these animals are useful as suitable bioindicators of pesticide contamination (Fossi et al., 1995; Sanchez et al., 1997; Sanchez-Hernandez and Walker, 2000). Chemical reactivation of blood ChE activity using pyridine 2-aldoxime methochloride (2-PAM) or spontaneous ChE reactivation in water-diluted samples has become an excellent index of exposure to OPs or CBs, respectively (McInnes et al., 1996; Parsons et al., 2000; Iko et al., 2003), although such a method is not always satisfactory in all species (e.g., Escartin and Porte, 1997). Recently, we have examined the in vitro reactivation of OP-inhibited BChE activity of *G. galloti* using 2-PAM, a known potent reactivator of phosphorylated ChE (Sanchez-Hernandez and Moreno Sanchez, 2002). We concluded that this was an acceptable method of diagnosis of OP exposure. In spite of the promising results we observed in the laboratory using reactivation techniques with 2-PAM in the lizard *G. galloti*, we felt that the use of this diagnostic tool still needed to be further investigated under field conditions.

The lizard *G. galloti* is of special interest in a conservation context. It is endemic to and present only on the Canary Islands and is listed as a "strictly protected species" by the Convention on the Conservation of European Wildlife and Natural Habitats (Bern, Switzerland, 1979) and by the Directive on the Conservation of Natural and Semi-Natural Habitats and of Wild Fauna and Flora (92/43/EEC, Annex IV). In an ecological context, this lizard species is represented by four subspecies distributed separately in four islands: *Gallotia galloti galloti* in the Island of Tenerife, *Gallotia galloti palmae* in La Palma, *Gallotia galloti gomerae* in La Gomera, and *Gallotia galloti caesaris* in El Hierro (Fig. 1). These lizards feed mainly on fleshy fruit and they play an important role as seed dispersers (Olesen and Valido, 2003). This study is part of a continuing investigation into the assessment of acute and chronic effects of OP/CB pesticides on wildlife, in particular lizards, of the Canary Islands. The aims of this field study were (1) to determine whether the massive use of pesticides on the Island of La Palma (Canary Islands) represents a hazard to lizard populations inhabiting landscapes surrounding agricultural areas there, and (2) to validate the use of reactivation techniques applied to plasma BChE activity for assessing OP and CB exposure. Exposure was evaluated through the measurement of plasma BChE activity levels and its reactivation after 2-PAM treatment (assessing exposure to OPs) or water dilution of the sample (assessing exposure to CBs). Body condition and hematocrit were also used to evaluate the physical condition of the lizards.

2. Materials and methods

2.1. Sampling

Adult lizards (*G. galloti palmae*) were collected in two agricultural areas (Fuencaliente and Tazacorte) and two reference areas (Los Llanos and Tigalate) on the Island

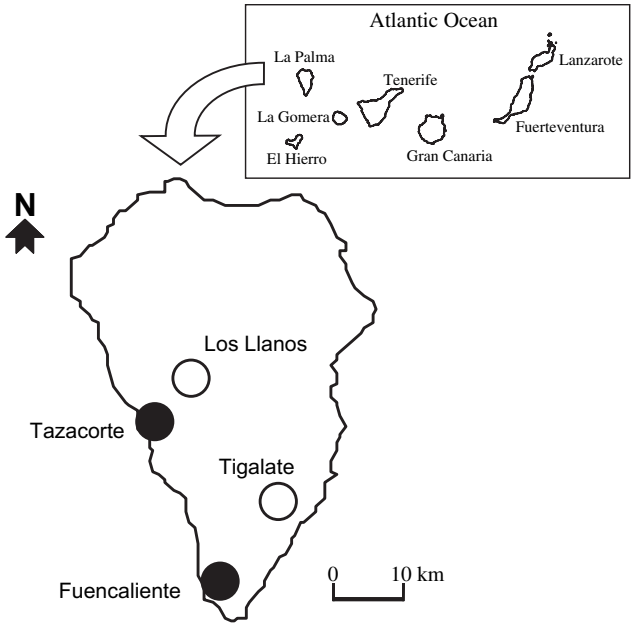

Fig. 1. Location of the sampling sites on the Island of La Palma (Canary Islands, Spain). Reference areas are shown by a white circle and the agriculture areas by a black circle. Sampling at Los Llanos and Fuencaliente was carried out in August 2002, whereas sampling at Tigalate and Tazacorte was performed in November 2002.

of La Palma (Fig. 1). Fuencaliente and Tazacorte are highly active agricultural areas producing bananas. The sampling campaign was performed two times: 290 lizards were collected from Los Llanos and Fuencaliente in August 2002, whilst 130 lizards were collected from Tigalate and Tazacorte in November 2002. Lizards were captured at midday using cages ($40 \times 15 \times 30$ cm) baited with pieces of fruit. Six to eight traps were randomly deployed at each sampling site, and were recovered 1 h later. This operation was repeated twice a day. Lizards were kept under dark conditions for a few minutes to reduce stress due to capture. Each lizard was put on ice to reduce activity and a blood sample ($\sim$100 μl) was collected from the postorbital sinus using heparinized microcapillary tubes. Blood samples were immediately placed in portable refrigerators containing ice ($\sim$4 °C). Within 4 h after blood collection plasma was separated from the whole-blood sample by centrifugation (10,000 rpm for 5 min) using a hematocrit centrifuge. After hematocrit determination, plasma samples were frozen at −80 °C until needed for BChE assays. Sex, head–cloaca length and body weight were recorded prior to the animals being released; the latter two parameters were used to calculate a condition factor (CF) for each animal as proposed by Bagenal and Tesch (1987) and expressed as: 100 × body weight (g)/(length (cm)^3).

2.2. Butyrylcholinesterase assay

Plasma BChE activity was determined colorimetrically by the method of Ellman et al. (1961) with modifications for optimal assay conditions (Sanchez-Hernandez and Moreno, 2002). Briefly, plasma was preincubated for 2 min with 5,5′-dithiobis-2-nitrobenzoic acid (final concentration of 3×10^{-4} M) in 25 mM Tris–HCl, 1 mM $CaCl_2$ (pH 7.6) before the substrate butyrylthiocholine iodide (BuSCh) was added (final concentration of 2×10^{-3} M). Variations in optical density were recorded at 410 nm for 2 min at 25 °C using a Spectronic Genesis-5 (Spectronic Instruments, Rochester, NY) spectrophotometer. Plasma BChE activity was expressed as micromoles of substrate hydrolyzed per minute per millilitre of plasma using an extinction coefficient of 13,600 $cm^{-1}\,M^{-1}$. The reaction mixture—free of plasma—was periodically checked for non-enzymatic hydrolysis of BuSCh; no hydrolysis was observed.

2.3. Reactivation assay

Three aliquots of plasma per animal were used for testing reactivation of BChE activity using 2-PAM and deionised water (dH_2O). One aliquot was incubated with dH_2O (1/5 dilution factor) for 60 min at 25 °C. A second aliquot was incubated in the presence of 2-PAM (1/5 dilution factor obtaining a final concentration of 2×10^{-4} M) under the same conditions. A third aliquot of plasma was diluted 1/5 with dH_2O and immediately assayed for BChE activity. This latter aliquot was used as a control, and BChE activity was compared with that measured after dH_2O or 2-PAM incubation. The inter-assay coefficient of variation was less than 5%, and an increase of BChE activity above this value after 2-PAM or dH_2O treatments was accepted as significant. Increase of BChE activity in the presence of 2-PAM was indicative of inhibition by OP pesticides, whereas inhibition of BChE activity by CBs was assumed when activity increased after dilution of the plasma with dH_2O.

2.4. Data analysis

Analysis for effects of sex, head-cloaca length, body weight, sampling period (summer and autumn) and type of sampling area (agricultural and reference) on the response of plasma BChE activity, CF and hematocrit was performed using analysis of variance (Statistica software, version 6, StatSoft, Inc., Tulsa, OK, USA). Plasma BChE activity was logarithmically transformed, and hematocrit percentages were angularly transformed to normalize data. A level of probability below 0.05 was considered statistically significant.

Exposure to anti-ChE pesticides was evaluated according to two criteria: (1) a statistical criterion by which pesticide exposure was judged to have occurred when BChE activity was more than two standard deviations below the mean BChE activity of the reference group; and (2) a chemical criterion based on the in vitro reactivation of BChE activity after a 60-min incubation of plasma with 2-PAM or dH_2O. Significant differences in the number of lizards exposed to anti-ChE pesticides between the two agricultural sampling sites were examined using the Chi-square test after the percentage data were angularly transformed. Likewise, the percent of increase of BChE activity (angularly transformed data) after dH_2O or 2-PAM treatments was compared between Fuencaliente and Tazacorte using analysis of variance.

3. Results

3.1. Hematocrit and condition factor

Sampling site significantly influenced the hematocrit of lizards sampled in autumn (Table 1). Mean (±SD) hematocrit percentage was lower in the lizards collected at Tazacorte (25.7 ± 4.05 for males and 26.0 ± 3.86 for females) relative to that of lizards caught at Tigalate (35.6 ± 4.11 for males and 32.5 ± 5.72 for females). However, no significant variations were found in the hematocrit values of lizards collected at Fuencaliente

Table 1
Summary of univariate analysis of variance (ANOVA) of logarithmically transformed butyrylcholinesterase (BChE) activity, condition factor and angularly transformed hematocrit for *Gallotia galloti palmae*

Dependent variable	Source of variation[a]	Sum of squares	df	*F*	*P*
BChE activity	Sex	0.0098	1	0.573	0.4494
	Head–cloaca length	0.0001	1	0.006	0.9344
	Body weight	0.0003	1	0.021	0.8825
	Sampling period	0.3391	1	19.839	<0.0001
	Sampling site	0.5460	1	31.942	<0.0001
	Sampling period × sampling site	0.0175	1	1.024	0.3122
	Sampling period × sex	0.0176	1	1.030	0.3106
	Sampling site × sex	0.0231	1	1.353	0.2453
	Sampling period × sampling site × sex	0.0115	1	0.676	0.4112
	Error	0.0170	366		
Condition factor	Sex	1.517	1	8.46	0.0038
	Sampling period	21.468	1	119.63	<0.0001
	Sampling site	0.014	1	0.080	0.7771
	Sampling period × sampling site	0.682	1	3.800	0.0519
	Sampling period × sex	0.165	1	0.920	0.3378
	Sampling site × sex	0.000	1	0.000	0.9655
	Sampling period × sampling site × sex	0.001	1	0.000	0.9573
	Error	72.316	403		
Hematocrit	Sex	1.817	1	0.230	0.6311
	Head–cloaca length	0.923	1	0.117	0.7321
	Body weight	7.700	1	0.978	0.3232
	Sampling period	5.911	1	0.751	0.3867
	Sampling site	385.60	1	48.99	<0.0001
	Sampling period × sampling site	332.49	1	42.24	<0.0001
	Sampling period × sex	3.276	1	0.416	0.5192
	Sampling site × sex	4.699	1	0.597	0.4402
	Sampling period × sampling site × sex	36.02	1	4.576	0.0330
	Error	7.871	374		

[a] Categorical independent variables: sex = males and females (sex-undetermined individuals were not included in the statistical analysis); sampling period = summer and autumn; sampling site = reference and agricultural areas.

relative to those collected at Los Llanos. Table 2 summarizes the biometric parameters of lizards from the reference sites and agricultural sites. In general, the females were significantly smaller ($F_{1,103} = 57.81$, $P < 0.0001$) and weighed less ($F_{1,102} = 48.72$, $P < 0.0001$) than males. The CF of the lizards was significantly ($F_{1,403} = 119.6$, $P < 0.0001$) influenced by the sampling period (Table 1). Mean CF of lizards collected in summer was significantly lower than that of lizards collected in autumn (Table 2); however, no differences were found between the CF of lizards from the agricultural areas respective to the CF of lizards from the reference area, irrespective of the sampling period (Table 1).

3.2. Levels of BChE activity

Mean (±SD) plasma BChE activity of non-exposed lizards varied from 3.07 ± 1.22 μmol min^{-1} ml^{-1} for lizards from Los Llanos to 3.61 ± 2.57 μmol min^{-1} ml^{-1}

Table 2
Mean (±standard deviation) values of the biometric parameters and condition factor (CF[a]) of lizards caught at each sampling site on the Island of La Palma

Sampling site	Sampling period	*N*	Sex	Head–cloaca length (mm) ± SD	Body weight (g) ± SD	CF ± SD
Reference areas						
Los Llanos	Summer	45	Males	99.4 ± 9.2	29.8 ± 10.7	2.89 ± 0.42
		32	Females	89.6 ± 5.2	19.4 ± 4.0	2.67 ± 0.33
Tigalate	Autumn	9	Males	96.7 ± 9.4	32.5 ± 9.0	3.56 ± 0.54
		20	Females	82.3 ± 6.5	19.1 ± 5.1	3.45 ± 0.45
Agricultural areas						
Fuencaliente	Summer	148	Males	98.6 ± 10.0	30.0 ± 10.2	3.01 ± 0.44
		65	Females	86.1 ± 5.6	18.1 ± 3.9	2.80 ± 0.32
Tazacorte	Autumn	51	Males	104.5 ± 8.0	42.3 ± 12.0	3.46 ± 0.50
		50	Females	88.5 ± 7.1	22.5 ± 5.3	3.35 ± 0.40

[a] The CF is calculated as the ratio between body weight and length: 100 × body weight (g)/(length $(cm)^3$).

Table 3
Plasma butyrylcholinesterase (BChE) activity (μmol min^{-1} ml^{-1} of plasma) in the lizard *Gallotia galloti palmae*

Sampling site	Sampling period	Sex	Mean ± SD	Min	Max
Reference areas					
Los Llanos	Summer	Males	3.07 ± 1.22	1.41	7.31
		Females	3.00 ± 1.09	1.40	4.89
Tigalate	Autumn	Males	3.61 ± 2.57	2.33	8.84
		Females	4.06 ± 1.30	1.73	6.38
Agricultural areas					
Fuencaliente	Summer	Males	2.06 ± 1.04	0.41	5.76
		Females	1.92 ± 0.84	0.59	4.13
Tazacorte	Autumn	Males	2.93 ± 1.16	1.40	6.84
		Females	2.83 ± 0.98	0.37	4.50

for lizards from Tigalate. No significant difference ($F_{1,83} = 2.21$, $P = 0.1407$) was found in BChE activity between both sexes for animals collected at the reference sites (Table 3). BChE activity (logarithmically transformed data) was significantly lower in the lizards collected from the agricultural areas relative to those from the reference areas (Fig. 2; Table 1). In addition, the enzyme activity was significantly different between the sampling periods (Table 1), with the highest BChE activities measured in lizards sampled in autumn (Table 3). However, the statistical comparison of the mean enzyme activities among sampling sites was not sufficient for making conclusions related to the impact of anti-ChE pesticides on these animals. Therefore, we calculated a diagnostic threshold value (mean enzyme activity minus two standard deviations) from the logarithmically transformed BChE activity of the reference groups in order to detect individuals from the agricultural areas with depressed enzyme activity. This diagnostic threshold had a value of 0.15 for the group of lizards from Los Llanos and 0.22 for the group from Tigalate (Fig. 3). The percent of lizards with plasma

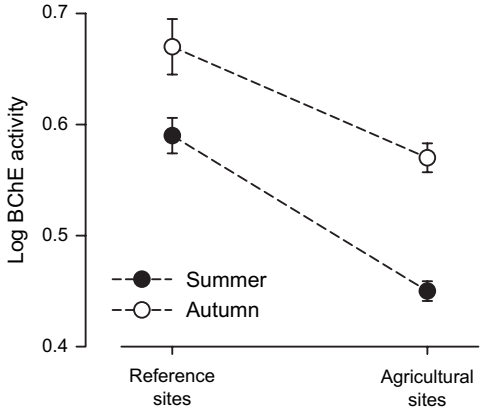

Fig. 2. Significant differences ($P < 0.001$) of plasma butyrylcholinesterase (BChE) activity (log transformed data) of the lizards collected from the reference and agricultural sites in both sampling periods (August and November). Plotted values are means ± 1 SE.

BChE activity below the threshold value sampled at Fuencaliente (23%) was significantly higher ($\chi^2 = 11.64$, df = 1, $P < 0.001$) than that of lizards from Tazacorte (7%). No significant variations were observed in the percent of males and females having BChE depressed for each agricultural sampling sites (Fig. 4A).

3.3. Reactivation of BChE activity

The reactivation of BChE activity using 2-PAM or dH_2O was not possible for all samples because of plasma volume limitations. A total of 249 samples from the agricultural areas (n = 180 from Fuencaliente; n = 69 from Tazacorte) were assayed for plasma BChE reactivation using 2-PAM and dH_2O (Fig. 4B; Table 4). Positive reactivation of BChE activity was found in most of the samples from the two agricultural areas after incubation with 2-PAM or dilution with dH_2O. The number of samples from Fuencaliente in which plasma BChE activity increased after 2-PAM treatment or dH_2O dilution of the sample was significantly higher ($\chi^2 = 30.99$, df = 1, $P < 0.0001$) than that from Tazacorte (Fig. 4B). BChE activity of five lizards from Fuencaliente and two lizards from Tazacorte did not increase after the reactivation treatment although BChE activity for these lizards was below the diagnostic threshold value. Plasma BChE activity for lizards collected from the reference site was also measured after 60-min incubation with 2-PAM or dH_2O to evaluate changes in enzyme activity greater than 5% of control enzyme activity. No significant increase of BChE activity was found in the plasma of lizards caught at the reference sites.

Most of the samples assayed for reactivation showed an increase of BChE activity when they were diluted with dH_2O (Table 4). The reactivation protocol used in this study enabled us to determine whether inhibition of plasma BChE activity was caused by both OP and CB pesticides. This could be diagnosed when the percent of increase of BChE activity after 2-PAM treatment of a sample was higher than that obtained after dH_2O dilution of the same sample. Thirty-nine lizards from Fuencaliente and seven from Tazacorte had an increase of BChE activity after 2-PAM and dH_2O incubations (Table 4).

Plasma samples from Tazacorte showed percentages of increase of BChE activity that varied from 5.13 to 44.4% when plasma was diluted with dH_2O, and from 5.34 to 53.2% when it was incubated in the presence of 2-PAM. However, BChE activity of the lizards collected at Fuencaliente showed a higher level of reactivation when plasma was diluted with dH_2O (up to 65% of increase) than when it was incubated in the presence of 2-PAM (<25%). Indeed, samples from Fuencaliente had a higher ($F_{1,168} = 13.50$, $P < 0.001$) percent of BChE increase (angularly transformed data) after dH_2O

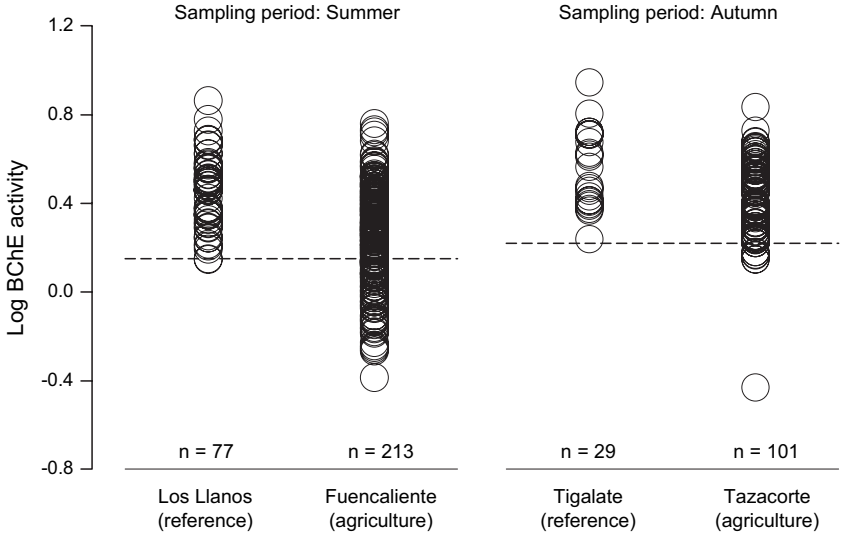

Fig. 3. Plasma butyrylcholinesterase (BChE) activity (log transformed data) of the lizard *Gallotia galloti palmae* from two agricultural sites (Fuencaliente and Tazacorte) and from two reference sites (Los Llanos and Tigalate) on the Island of La Palma. The dashed lines indicate the diagnostic threshold values (log BChE = 0.15 for the sampling in summer; log BChE = 0.22 for the sampling in autumn) which are the mean log BChE activity of the reference groups minus two standard deviations. Numbers below symbol columns indicate the number of plasma samples assayed for BChE activity; they included plasma samples males, females and sex-undetermined individuals.

treatment than samples from Tazacorte (Fig. 5). However, the females from Tazacorte whose BChE activity showed positive reactivation to 2-PAM treatment had an increase of BChE activity significantly lower ($F_{1,67} = 4.50$, $P < 0.05$) respective to males from Tazacorte or individuals (males and females) from Fuencaliente (Fig. 5).

4. Discussion

A high number of lizards from agricultural areas, especially Fuencaliente, showed evidence of exposure to cholinesterase-inhibiting pesticides. Plasma BChE activity of these lizards increased after incubation of the samples with 2-PAM and/or dilution in dH_2O. There is a sufficient diagnostic evidence that OP and CB pesticides were involved in the depression of BChE activity. In other words, although chemical analysis of anti-ChE pesticide residues was not performed in the environmental matrices (soil, vegetation) or in the whole organism, the chemical reactivation of BChE activity was considered sufficient to give a reliable assessment of pesticide exposure. In our study, spontaneous reactivation of BChE activity after water dilution of plasma took place in most of the samples collected from the agricultural sites (Table 4). These data suggest that CB pesticides could be the most important class of pesticides being applied to banana crops. We further speculate that carbofuran might be the CB responsible for plasma BChE inhibition because empty containers of this pesticide (Furadan® 20) were observed adjacent to the agricultural sampling sites. Carbamylated ChE is rapidly recovered, and field detection of ChE inhibition by this class of pesticides is often the result of multiple CB applications. Frequent applications of CB pesticides could take place in our study sites; the favourable weather conditions of the Canary Islands help maintain populations of foliage insects and agricultural nematodes in the banana crops, and pesticide applications occur frequently. On the other hand, the percentage of lizards with BChE activity depressed—according to the statistical criterion—was higher in the group caught at Fuencaliente site (23%) relative to the group collected at Tazacorte site (7%). Furthermore, 88% of plasma samples of lizards from Fuencaliente showed BChE reactivation after 2-PAM or dH_2O treatments as opposed to the 58% of samples from Tazacorte site. Finally, increases of BChE activity after chemical reactivation were generally much higher in the samples from Fuencaliente respective to those from Tazacorte (Fig. 5). All these considerations induce us to suggest Fuencaliente as the agricultural area where pesticide applications had a higher impact on lizards, at least in terms of sublethal exposure to anti-ChE pesticides.

Mean BChE activity in this Gallotia subspecies was two-fold lower than that found from *G. galloti galloti*, the endemic subspecies from the Island of Tenerife (Fig. 1), in which we have measured a mean ($\pm$SD) BChE activity of 6.28 ± 1.9 μmol min^{-1} ml^{-1} for males and 6.61 ± 0.7 for females (Sanchez-Hernandez, 2003). This suggests that BChE activity in non-exposed lizards can be substantially different among the four subspecies of *G. galloti*, and it should be taken into account when

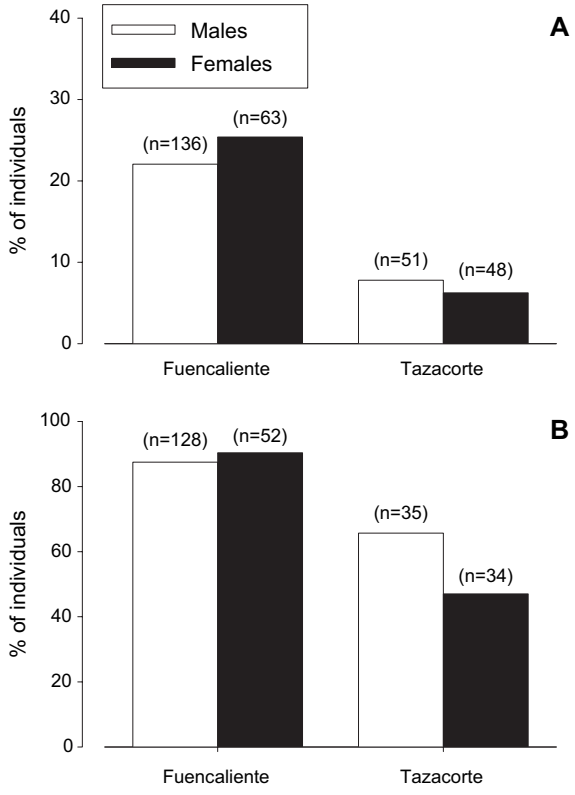

Fig. 4. (A) Proportion of male and female lizards collected from the agricultural sites with plasma butyrylcholinesterase (BChE) activity below the statistic diagnostic threshold (statistical criterion). Numbers in parentheses indicate the total number of samples assayed for enzyme activity. (B) Proportion of lizards that showed positive reactivation of plasma BChE activity after pyridine-2-aldoxime methochloride incubation or after dilution of the plasma with deionised water (chemical criterion). Numbers in parentheses indicate the total number of samples used for enzyme reactivation analysis. These values did not correspond with the number of samples used for BChE determinations because of plasma volume limitations.

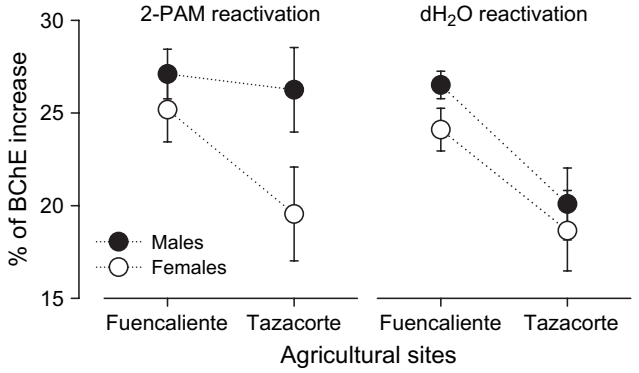

Fig. 5. Percentage of increase of plasma butyrylcholinesterase (BChE) activity (angularly transformed data) in the samples collected from the agricultural sites, after incubation (60 min at 25 °C) of plasma samples with pyridine-2-aldoxime methochloride (2-PAM) or deionised water (dH_2O). Plotted values are means ± 1 SE.

biomonitoring programs of pesticide pollution are carried out in different islands. BChE activity seemed to show a seasonal variation reaching higher values in summer (Table 3), although we have to consider that the reference site was not the same in both sampling campaigns (summer and autumn). There is abundant evidence that ChE levels in non-exposed animals vary as a function of ambient temperature, which is reflected in seasonal fluctuations of enzyme activity with maximum values during the summer (Hill and Murray, 1987; Edwards and Fisher, 1991; Thompson and Walker, 1994). Such a seasonal effect could account for the significant variation of plasma BChE activity found in the lizards collected from the two reference areas (Los Llanos and Tigalate).

Chemical reactivation of BChE activity employing 2-PAM and water dilution (i.e., spontaneous reactivation) resulted in a better method to diagnose exposure to anti-ChE pesticides than making comparisons of BChE activity levels between non-exposed and exposed groups. As clearly shown in Fig. 4, the real impact of anti-ChE pesticide applications on lizards may be underestimated when one only considers a statistical criterion (mean BChE activity of the reference group minus two standard deviations) for assessing OP/CB exposure. However, some methodological aspects should be considered when oximes are used for determining inhibition of ChE activity due to OPs. For example, the concentration of 2-PAM is critical to achieve full reactivation of phosphorylated ChE. Monserrat and Bianchini (2000) demonstrated that high concentrations of 2-PAM (3×10^{-4} M) caused inhibition of AChE activity in fish, crabs and eels. Likewise, this 2-PAM concentration induced non-enzymatic hydrolysis of the substrate in the reaction medium. Escartin and Porte (1997) were not able to reactivate the AChE activity of mussels (*Mytilus galloprovincialis*) experimentally exposed to the OP fenitrothion. They used 2-PAM concentrations of 5,

Table 4
Number and percentage of plasma samples that showed in vitro reactivation of butyrylcholinesterase (BChE) activity after treatment with pyridine-2-aldoxime methochloride (2-PAM) or after sample dilution with deionised water (dH_2O)

Site	N[a]	2-PAM		dH_2O		2-PAM and dH_2O	
		Number of positive reactivations	%	Number of positive reactivations	%	Number of positive reactivations	%
Fuencaliente	180	14	7.7	114	63.3	39	21.6
Tazacorte	69	13	18.8	20	29.0	7	10.1

[a] Total number of samples assayed for BChE reactivation.

10, and 20 mM, which could have been high enough to completely recover the phosphorylated AChE activity. Sanchez-Hernandez and Moreno (2002) reported an optimum 2-PAM concentration of 2×10^{-4} M in order to get a successful reactivation of phosphorylated plasma BChE activity in the lizard *G. galloti*. These studies suggest that a previous characterization of the reactivation technique using 2-PAM should be made for the species and ChE type under investigation before it is used as a diagnostic tool of OP exposure. We used a 2-PAM concentration we considered optimum because previous studies in our laboratory have demonstrated that maximum BChE reactivation is achieved without the associated problems of enzyme inhibition or spontaneous hydrolysis of the substrate (Sanchez-Hernandez and Moreno, 2002).

In a recent field study, Sanchez-Hernandez (2003) suggested that chemical reactivation of BChE activity in the presence of 2-PAM should be used as a specific index of OP exposure in natural environments where the subspecies *G. galloti galloti* from the Island of Tenerife could be used as a bioindicator. In the present study however, we were able to differentiate between OP and CB pesticide exposure in a field scenario: it was possible to detect BChE inhibition by both OP and CB pesticides in a single sample (Table 4). However, we have found some unexpected observations. Two samples collected from the agricultural sites did not show positive reactivation of BChE activity although they were initially below the diagnostic threshold value. McInnes et al. (1996) similarly had blood samples from nestling songbirds (*Agelaius phoeniceus*, *Passer domesticus* and *Molothrus ater*) with ChE activity below the diagnostic threshold value calculated from non-exposed birds, and none showed significant reactivation after 2-PAM treatment. The authors suggested that chemicals other than OPs could account for plasma ChE depression in those birds. We cannot explain this observation, but it is well known that phosphorylated ChE can undergo an aging phenomenon. Such aging occurs when the OP loses an alkyl group once it binds to the active site of the enzyme; thus the enzyme remains permanently inactivated. Under these conditions, reactivation of ChE is not possible, either spontaneously or with reactivating agents (such as 2-PAM; Thompson and Walker, 1994). Several in vitro and in vivo studies have shown that aging of ChE is dependent on the time since exposure (Soler-Rodriguez et al., 1998; Thompson et al., 1991). Stansley (1993) found an incomplete AChE reactivation in fish and birds after 2-PAM treatment, which he attributed to the aging of phosphorylated AChE. The lack of reactivation in some of our 2-PAM treated samples, which originally had BChE activity below the diagnostic threshold value, might be due to an aging of the enzyme, but our study also emphasizes the need to investigate whether the in vitro efficacy of 2-PAM to reactivate phosphorylated BChE is dependent on the time since enzyme inhibition occurred.

We examined the animals' condition factor and hematocrit to find evidence of chronic effects from pesticide exposure on the physiological status of lizards inhabiting the agricultural areas. In general, an increase of hematocrit can be related to higher oxygen demands, whereas a reduction is an index of anemia, which may reflect a nutritional deficiency, bacterial infection, or exposure to pollutants (Ots et al., 1998). For example, a reduction of hematocrit was reported in birds inhabiting environments polluted by heavy metals (Grue et al., 1986), and in fish experimentally exposed to the herbicide molinate (Sancho et al., 2000). The low haematocrit values measured in the blood of lizards collected at Tazacorte in comparison with those from the reference area (Tigalate) could be related to pesticide exposure, and in turn, to a nutritional deficiency or could be related to the toxicity that OPs can exert on the immune system. It has been recognized that OP pesticides alter, directly or indirectly, the immune systems of vertebrates and increase their susceptibility to infectious diseases (Galloway and Handy, 2003). Nevertheless, immunological indices other than hematocrit (e.g., total leukocyte counts) are needed to give further evidence of a cause–effect relationship between pesticide exposure and immunotoxicity in our study animals. The CF is a general stress index that can provide information about the nutritional status of an organism. In fish, variation of this factor has been related to polluted environments. An increase of CF was found in brown bullhead (*Ictalurus nebulosus*), English sole (*Parophrys vectulus*) and perch (*Perca fluvialis*) from environments contaminated by organic pollutants (Van der Oost et al., 2003). We have also found an increase in the mean CF value in lizards collected in autumn respective to those sampled in summer, irrespective of the sampling site. However, it seemed to be having no direct link between toxic effects from pesticide exposure and CF of the lizards because no significant variations were observed in the CFs between lizards from the agricultural areas and lizards from the reference areas.

In conclusion, our results show that lizards from the two agricultural sites are currently exposed to anti-ChE pesticides, and pesticide applications appear to be more intensive in the Fuencaliente site than in Tazacorte site. Although mortality of lizards was not observed throughout the sampling periods, OP and CB applications in the agricultural areas could represent a hazard to lizard populations in view of the evident exposure they are receiving based on our results of BChE reactivation. Such information would be useful to the regulatory agencies of the European Union in order to make decisions about pesticide management in areas of high ecological interest such as the Canary Islands. Our results should be considered as a first phase in a scheme

of environmental risk assessment from agricultural pesticide applications in the islands. However, the sublethal chronic effects and population level impacts, if any, on our study species need to be determined.

Acknowledgements

The authors thank Bruce Pauli for his valuable comments on a drafted version of the manuscript, and farmers from the Island of La Palma for their co-operation. This study was funded by the Cabildo Insular de La Palma (Canary Islands, Spain) and the University of Castilla-La Mancha (Toledo, Spain).

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