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Subchronic exposure to chlorpyrifos affects energy expenditure and detoxification capacity in juvenile Japanese quails

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HIGHLIGHTS

- Quails chronically exposed to chlorpyrifos respond through biochemical and systemic modifications.
- Esterase activities increases with age in control groups but remains unchanged in exposed birds.
- Glucuronic acid output increases in individuals exposed to chlorpyrifos.
- Exposure to chlorpyrifos leads to high energy costs in quails, affecting their aerobic performance.

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ABSTRACT

Effects of pesticides on non-target organisms have been studied in several taxa at different levels of biological organization, from enzymatic to behavioral responses. Although the physiological responses may be associated with higher energy costs, little is known about metabolic costs of pesticide detoxification in birds. To fill this gap, we exposed orally (diet) 15-d old *Coturnix coturnix japonica* individuals to sublethal doses of chlorpyrifos (10 and 20 mg active ingredient/kg dry food) for four weeks. Carboxylesterase (CbE), butyrylcholinesterase (BChE) and acetylcholinesterase (AChE) activities were periodically measured in multiple tissues along with measurements of resting (RMR) and maximum metabolic rates (M_{sum}). Furthermore, glucuronic acid in bird excreta was also assessed at the end of the trial. While CbE and BChE activities were inhibited by chlorpyrifos in all tissues during the third and fourth weeks following pesticide treatment, AChE activity was unaffected. At this sampling times, both M_{sum} and RMR expansibility decreased. These results suggest that the exposure to chlorpyrifos caused a negative effect on aerobic performance. Additionally, excretion rate of glucuronic acid was up to 2-fold higher in the 20-mg/kg group than in the control and 10-mg/kg chlorpyrifos groups. The inhibition of CbE and BChE activities corroborated that these enzymes are fulfilling their role as bioscavengers for organophosphate pesticides, decreasing its concentration and thus protecting AChE activity against inhibition by chlorpyrifos.

1. Introduction

Most of authorized pesticides are neurotoxins, which cause in non-target organisms behavioral changes such as reduced food intake, lower reproductive success and poorer ability to avoid predation (Walker, 2003; Boatman et al., 2004). Among these neurotoxins, carbamates (CB) and organophosphate (OP) compounds are widely used in agriculture (EPA, 2011) and chlorpyrifos (O,O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate) is one of the most widely used OP insecticides effective against a broad spectrum of insect pests (EPA, 2011). Chorpyrifos can be absorbed easily through the gastrointestinal mucosa, lung epithelium, and skin (Testai et al., 2010). It is nearly insoluble in water (2.6 mg/L

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at 25 °C) and highly soluble in most of organic solvents (Log K_{OW} = 4.24). This OP insecticide degrades slowly in soil under both aerobic and anaerobic conditions, displaying a half-life of 60–120 days (Testai et al., 2010). Concentrations of chlorpyrifos normally reported for agricultural soils after soil-surface applications ranged between 0.2 and 58.8 mg/kg (reviewed in Racke, 1993).

The mechanism for acute toxicity of OP pesticides is found in the irreversible inhibition of acetylcholinesterase (AChE EC 3.1.1.7), which is a key enzyme in the function of the nervous and neuromuscular systems (Thompson and Richardson, 2004). This interaction is however highly effective with the 'oxon' metabolite of the OP, which is formed through oxidative desulfuration catalyzed mainly by the cytochrome P450-dependent monooxygenases (Hodgson, 2010). In fact, these oxygen-analog metabolites display a toxic potency two-three orders of magnitude higher than their parent compounds because they have a higher affinity by the active site of serine hydrolases such as AChE (Chambers et al., 2010). Accordingly, inhibition of brain AChE activity has been the most widely used biomarker for diagnosing harmful or simple exposure to OPs in a wide variety of terrestrial and aquatic organisms (Fulton and Key, 2001; van der Oost et al., 2003; Domingues et al., 2010; Auon et al., 2014). Nevertheless, other serine hydrolases such as butyrylcholinesterase (BChE, EC 3.1.1.8) and carboxylesterase (CbE, EC 3.1.1.1) have been increasingly used as complementary indicators of pesticide exposure (Sogorb et al., 2007; Wheelock et al., 2008). Several studies have reported that these esterase activities are more sensitive to inhibition by OP insecticides than brain AChE activity (Wheelock et al., 2008; Sanchez-Hernandez, 2001). Therefore, phosphorylation of these esterases is considered an efficient stoichiometric mechanism of OP detoxification, which protects nervous AChE activity against inhibition by OPs (Russel and Overstreet, 1987). Beside the esterase-mediated detoxification of OPs, CYP450s may also catalyze the dearylation of chlorpyrifos to yield diethylthiophosphate and 3,5,6-trichloro-2-pyridinol, which is conjugated with endogenous molecules (glucuronic acid and sulfate) to facilitate its excretion in the form of water-soluble conjugated metabolites (Jokanovic, 2001; Testai et al., 2010). It has been suggested that the glucuronidation pathway is a major route of detoxification in vertebrates (Clarke et al., 1991; Jakubas et al., 1993; Guglielmo et al., 1996).

Maintenance costs are essential to life continuity and include standard maintenance (e.g. basal energy costs and tissue reparation) and activity maintenance (necessary energy for foraging, circadian rhythms and digestion), while the energy used for production can be divided between growth and reproduction (Weiner, 1992). When energy availability is limited, maintenance and production functions are competing, so when maintenance costs become priority, energy spent on higher activity and reproduction should be lowered (McNab, 2002). In this situation, animals exposed to chemical stressors such as plant secondary compounds or pollutants may experience energetic costs caused by the development of physiological processes to reduce the impact of stressors. In turn, stressors can also decrease energy assimilation efficiency (Karasov and Martinez del Rio, 2007), alter environmental energy accessibility (Hopkins et al., 2004) and affect activity levels, or performance physiology (Raimondo et al., 1998). Despite that energy cost related to pesticide exposure have been studied in fish and some lizards (Beyers et al., 1998; DuRant et al., 2007), as far as we know, there are no studies that relate OP pesticides exposure with energetic metabolism. In order to fill this gap of knowledge, we studied the interplay between pesticide exposure, esterases activities and energy expenditure rates in juvenile Japanese quails (Phasianidae: *Coturnix coturnix japonica*).

The aim of this work was to examine the energetic cost associated to detoxification and its consequences on the activity of CbE, BChE and AChE in three different tissues of chlorpyrifos- exposed Japanese quails. *Cotournix c. japonica* is a well-suited model species for the study of pesticide risk assessment in birds (Zhang et al., 2015) because its fast growth rate, small size, early sexual maturity, easy to maintain in the laboratory, and relatively small food consumption (Slama et al., 1996). It has been also reported that this species respond physiologically to chlorpyrifos exposure (Auon et al., 2014) and exhibits metabolic plasticity during the first days after hatching (Rønning et al., 2009). Considering that pesticide exposure would cause energy and nutrients expenditure associated to detoxification costs, we hypothesized that chlorpyrifos would cause an imbalance in energy and matter budgets. As a consequence of such imbalance, we hope to find (1) a decrease in the body mass of developing quails due to the use of a greater amount of energy spent on detoxification, (2) a higher resting metabolic rate (RMR) and a lower maximum cold-induced metabolic rate (M_{sum}) (3) a smaller size in quails organs due to the lower amount of energy used in tissue production, (4) an inhibition in CbE and BChE activities, as a non-catalytic and stoichiometric detoxification system of chlorpyrifos, and finally (5) an increase in the excreted glucuronic acid.

2. Materials and methods

2.1. Reagents

Chemicals used in kinetic enzyme assays, i.e., α-naphthyl acetate (α-NA), 4-nitrophenyl acetate (4-NPA), butyrylthiocholine iodide (BTCI), acetylthiocholine iodide (ATCI), 4-nitrophenol, Fast Red ITR salt and 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), and the reagents used for glucuronic acid assays, i.e., 3-phenylphenol and standard glucuronic acid, were purchased from Sigma–Aldrich (Santiago, Chile). The substrates α-NA and 4-NPA were prepared in ethanol and kept at 4–5 °C. Chlorpyrifos was purchased in the emulsifiable formulation Lorsban® 4E (48%, active ingredient) through CALS Company (Santiago, Chile).

2.2. Exposure set up

Fifty-four Japanese quails (15-d old) were purchased from a commercial hatchery (Santiago, Chile), and randomly assigned into three experimental groups (n = 18) and fed with commercial Champion Codorniz Starter base food (24% protein, 5.5% fiber, 3.5% ether extract, 14% humidity). Because the development time was crucial in this study, individuals were immediately subjected to experimental treatments, upon arrival in the laboratory. Sublethal effects of chlorpyrifos were assessed using two nominal concentrations, i.e., 10.1 and 20.2 mg active ingredient/kg of food. It has been reported recently that chlorpyrifos caused several clinical and pathological alterations in internal organs of quails at sublethal concentrations (Auon et al., 2014). Thus, it was assumed that concentrations in our study would minimize adverse effects and prevent the risk for ill. The pesticide was sprayed on 1 kg of dried commercial food to yield the nominal concentrations. Two groups were fed with chlorpyrifos-containing diets for four weeks and a third group was used as control, which received a pesticide-free food. Considering the mean food intake of quails in the 35-days period (361.3 ± 11.3 and 385.3 ± 45.7 g for both pesticide treatments, respectively) and the nominal chlorpyrifos concentrations, we estimated that the total doses were 36 and 77 mg active ingredient/kg body mass for both experimental treatments, respectively. These chlorpyrifos doses represented 7.3 and 15.7% of the median lethal concentration in quails (492 mg/kg), which was defined by Hill and Camardese (1986) as the concentration of chlorpyrifos in

a 5-days ad libitum diet calculated to kill 50% of the test population. An exposure period of four weeks was selected because Japanese quails reach sexual maturity at 45-days old, when the egg laying begins. In consequence, we avoided the potential increases of metabolic rates associated with reproduction. All groups were kept under a constant temperature and light regime [25 °C, 12 h: 12 h (light:dark)]. Every week, 12 individuals (4 birds per treatment) were sacrificed by CO_2 exposure, their body mass recorded, and dissected abdominally to isolate the organs of interest that were weighed. Intestines were flushed with ice-cold saline solution (0.9% NaCl), measured (0.1 cm), weighed (0.0001 g) and finally frozen and kept at −80 °C until biochemical assays. The rest of organs were weighted and kept at −80 °C. Blood samples (75 μL) were taken each week from the brachial vein of individuals from each of the three treatments, blood samples were transferred to heparinized microtubes and kept cold (4 °C), then centrifuged (5000 rpm, 5 min, 4 °C) to collect the plasma, which was frozen at −80 °C.

2.3. Metabolic rates

Metabolic rates were estimated as the oxygen consumption (VO_2) using standard flow-through respirometry methods. For resting metabolic rate (RMR), birds were weighed, placed in a dark metabolic chamber (2 L), and then placed in a controlled temperature cabinet (Sable Systems, Henderson, Nevada) at a constant temperature (Ta = 30 ± 0.5 °C). The metabolic chamber received dried air at 500 mL min^{-1} from a mass flow controller and through Bev-A-Line tubing (Thermoplastic Processes Inc.). The excurrent air passed through columns of Driedrite, CO_2-absorbent granules of Baralyme, and Drierite before passing through an O_2-analyzer, model Fox Box (Sable System, Henderson, Nevada) calibrated with a known mix of oxygen (20%) and nitrogen (80%) that was certified by chromatography (INDURA, Chile). All measurements were made during the resting phase (between 18:00 and 07:00 h). Because water vapor and CO_2 was scrubbed before entering the O2 analyzer, oxygen consumption was calculated as VO_2 = [FR*60*(Fi O_2 – Fe O_2)]/(1-Fi O_2), where FR is the flow rate in ml/min after STP correction, and Fi and Fe are the fractional concentrations of O_2 entering and leaving the metabolic chamber, respectively.

Summit metabolism (M_{sum}) was determined in a He–O_2 (80-20%, INDURA, Chile) atmosphere (Rosenmann and Morrison, 1974) at −15 ± 2 °C, following a similar protocol used for RMR. Measurements were stopped when the reduction of VO_2 was evident after a visual inspection of the data. To corroborate that animals were hypothermic, body temperature (Tb) was checked with and intra rectal thermocouple (±0.1 °C) after each measurement. Only measurements with hypothermic birds were considered (e.g., Tb < 35 °C). Output from oxygen analyzer (%) and flow meter were digitalized using a Universal Interface II (Sable Systems) and recorded on a personal computer using EXPEDATA data acquisition software (Sable Systems).

2.4. Esterase activity determination

Tissues were thawed and the whole small intestine was homogenized in 20 volumes of 0.9% NaCl for 30 s at 24,000 rpm using an Ultra Turrax T25 homogenizer (Janke and Kunkel, Breisgau, Germany) at maximum setting. The homogenates were centrifuged at 5000 rpm for 10 min at 4 °C. Carboxylesterase activity was spectrophotometrically determined with a microplate reader Thermo Scientific Multiskan GO at 25 °C, using α-NA and 4-NPA as substrates. Specific activity was expressed as nmol min^{-1} mg^{-1} total protein. Total protein was quantified following the Bradford method (1976) using bovine serum albumin as standard. Carboxylesterase activity using α-NA was determined as described in Thompson (1999). Reaction medium (250 μL, final volume) contained Tris–HCl 0.1 M (pH 7.4) and α-NA 20 mM, and was incubated for 10 min at 25 °C with the sample. The formation of α-naphthol was stopped by adding 50 mg Fast Red ITR 0.1% in SDS 5%/Triton X-100 5% mixture. Solutions were kept for 30 min at 25 °C in darkness. The absorbance of the Napthol-Fast Red ITR complex was read at 530 nm. The specific activity was calculated using a molar extinction coefficient of 14 × 10^3 M^{-1} cm^{-1} for the naphtol-Fast Red ITR complex. Carboxylesterase activity using 4-NPA was determined following Chanda et al. (1997) method. Reaction medium (250 μL, final volume) contained Tris–HCl 0.1 M (pH 7.4) with the intestine sample. Reaction was initiated by adding 10 μL of 4-NPA 20 mM. Reaction was stopped after 10 min by adding 50 μL SDS 2% in Tris 2%. Formation of 4-nitrophenol was monitored at 405 nm and quantified with a 4-nitrophenol standard curve. The specific activity was calculated using a molar extinction coefficient of 8.0 × 10^3 M^{-1} cm^{-1}. BChE and AChE activities were determined according to Wheelock et al. (2005) adapted by Ellman et al. (1961). Reaction medium (200 μL, final volume) was composed of 0.1 M Na phosphate buffer (pH 8.0) with 320 μM (final concentration f.c.) 5,5′-dithiobis-2-nitrobenzoic acid 10 mM and 10 μL of sample. The reaction was initiated by adding 60 mM BTCI (BChE) or ATCI (AChE) and the formation of the product was monitored for 8 min at 412 nm. Specific activity was calculated using a molar absorption coefficient of 8.6 × 10^3 M^{-1} cm^{-1}.

2.5. Glucuronic acid in excreta

The excreta from each quail collected in the fourth week after treatment was analyzed for glucuronic acid concentration. Glucuronic acid was measured following the colorimetric assay described by Blumenkrantz and Asboe-Hansen (1973) and adapted by Jakubas et al. (1993). Briefly, 100 mg of ground, lyophilized, excreta was vigorously mixed with 10 mL of 0.01 M borate buffer (pH ca. 9.5) with vortex followed by 30 min of centrifuge at 1000 rpm. The supernatant was filtered (#1 Whatman filter paper) and 20 μL was added into a culture tube and diluted with distilled water to 200 μL. The culture tube was placed in an iced water bath and 3 mL of 0.0125 M sodium tetraborate-sulfuric acid solution was added and mixed using a vortex and returned to the iced water bath. Tubes were then heated in a water bath at 100 °C for 10 min. After cooling, 20 μL of the 0.5% aqueous NaOH reagent 3-phenylphenol was added to one set of samples. For the blank sample, the reagent was replaced by 20 μL of 0.5% NaOH. Determinations were made in triplicate. A standard curve was made with known concentrations of glucuronic acid. Absorbance was measured at 520 nm in a Shimadzu UV Mini-1240 UV–Vis spectrophotometer.

2.6. Data analysis

The effect of chlorpyrifos on the body mass was examined using repeated measures of analysis of variance (RM ANOVA). This same test was performed to assess the effect of chlorpyrifos on oxygen consumption (RMR and M_{sum}). To assess the effect of chlorpyrifos on energy expenditure rates, a one-way ANOVA for each week of treatment was performed. To establish the variation of the organs size between the treatments, an analysis of covariance, with body mass as covariate was performed. In addition, a two-way ANOVA with organs mass expressed as % of body mass was performed. Finally, esterase inhibition (CbE-αNA, CbE-4NPA, BChE and AChE) was compared among treatments and weeks, with a two-way ANOVA. All ANOVAs were followed by the Tukey post-hoc test. We set the level of significance at α = 0.05. All data are shown as

mean ± standard error. To investigate whether the data distribution were adjusted or not to a normal distribution, a Kolmogorov–Smirnov test, followed by the Shapiro–Wilks test was performed. Statistical analysis and graphs were made using Statistica 7.0 (StatSoft 2004) and SigmaPlot 12.0 (2011 Systat Software, Inc.) software.

3. Results

3.1. Body mass

At the beginning of the experiment (week 0, age: 15 days old), mean (±SD) body mass was 60.8 ± 8.9 g, which increased up to 137.0 ± 8.4 at the last week (age: 45 ± 3 days). No significant differences were registered in body mass variation throughout the experiment for the three treatments (two-way ANOVA: $F_{(8,73)} = 0.23$; $p = 0.98$).

3.2. M_{sum} and RMR

Mass-specific RMR (mL O_2 hr^{-1} g^{-1}) was significantly different (RM ANOVA: $F_{(2,15)} = 6.39$; $p = 0.0098$), but no significant differences were found when we compared between treatments for each week (see Table 1 for statisticall details). Specifically, a lower mass-specific RMR after 4 weeks of treatment was observed. When total RMR (mL O_2 min^{-1}) was compared, a non-significant increase (RM ANOVA: $F_{(2,15)} = 2.75$; $p = 0.096$) was found in week 4. Besides, total RMR was significantly higher in treatment 2 than in control group after two weeks of treatment (Table 1). Total M_{sum}, but not mass-specific M_{sum} exhibited differences over time (RM ANOVA: $F_{(2,3)} = 11.93$; $p = 0.037$; and $F_{(2,3)} = 5.17$; $p = 0.1$ respectively). Also, during the second week, both treatments exhibited higher M_{sum} values than control group although in the cases of mass-specific M_{sum} differences were in the limit of significance (Table 1). Besides individuals exhibited a decrease in M_{sum} after four weeks of exposure to chlorpyrifos in both treatments, but differences were only marginal.

3.3. Metabolic expansibility

ME = M_{sum}/RMR values did not present significant differences after quails were exposed for four weeks to the pesticide compared to week 2 (RM ANOVA: $F_{(2,3)} = 4.97$; $p = 0.11$). In week 4, ME values were significantly lower in both treatments with pesticide, compared to control (Table 1). Despite this, we observed that the ME value increased in the control group towards the end of the experiment (M_{sum4}/RMR_4), whereas decreased in both pesticide treatments (10 mg/kg: 55.8% decrease, $F_{(1,4)} = 17.64$; $p = 0.014$; 20 mg/kg: 67.9% decrease, $F_{(1,3)} = 16.59$; $p = 0.005$).

Table 2
Summary of two-way ANOVA for organ mass of quails orally exposed to chlorpyrifos.

Organ	Source of variation[a]	F	df	p
Liver	Time of exposure	18.83	3.37	<0.001*
	Concentration	0.48	2.37	0.62
	Time of exposure*Concentration	2.5	6.37	0.039*
S.I.	Time of exposure	21.4	3.37	<0.001*
	Concentration	1.83	2.37	0.17
	Time of exposure*Concentration	1.67	6.37	0.16
L.I.	Time of exposure	15.02	3.37	<0.001*
	Concentration	5.15	2.37	0.01*
	Time of exposure*Concentration	2.87	6.37	0.021*
Kidney	Time of exposure	1.87	3.37	0.15
	Concentration	1.04	2.37	0.36
	Time of exposure*Concentration	0.66	6.37	0.68
Gizzard	Time of exposure	14.39	3.37	<0.001*
	Concentration	5.94	2.37	<0.01*
	Time of exposure*Concentration	1.38	6.37	0.25
Heart	Time of exposure	11.16	3.37	<0.001*
	Concentration	1.04	2.37	0.36
	Time of exposure*Concentration	1.52	6.37	0.2
Cloaca	Time of exposure	6.92	3.37	<0.001*
	Concentration	5.69	2.37	<0.01*
	Time of exposure*Concentration	0.97	6.37	0.46

[a] Categorical independent variables: time of exposure = 1, 2, 3 and 4 weeks; treatment = 0, 10 and 20 mg/kg dry foods.

3.4. Organ sizes

Except for the kidneys, all organs showed significant differences in at least one of the considered factors (time of exposure, concentration, and time of exposure × concentration) (Table 2). The organs that were significantly affected considering the interactions were solely liver ($F_{(6,37)} = 2.5$; $p = 0.039$) and large intestine ($F_{(6,37)} = 2.87$; $p = 0.021$) (Fig. 1).

3.5. Esterase activity

Two-way ANOVA revealed that time of exposure, pesticide concentration and the interaction between these two factors varied with both the esterase type and the tissue (Table 3). To show in a clearer way these effects, *post hoc* Tukey test on pooled data according to time of exposure revealed the highest levels of CbE-4NPA activity in the fourth week of treatment in the small intestine and liver, and in the last two weeks in plasma (Fig. 2). Besides, CbE-αNA and BChE activities were highest in the small intestine and plasma after four weeks of treatment (Figs. 2 and 3). However, liver CbE-αNA and BChE activities remained unchanged (Figs. 2 and 3). Likewise, the highest levels of AChE activity were found in the fourth week for small intestine, although this esterase remained unaltered in liver (Fig. 3). Nevertheless, the *post hoc* analyses using all treatments (time of exposure × concentra-

Table 1
Summary of one-way ANOVA for RMR, M_{sum} and ME (mean ± SE). Subindex in RMR and M_{sum} correspond the week in which measurements were performed.

	Control	10 mg/kg	20 mg/kg	F	Df	p
RMR_2 (mL O_2 min^{-1})	1.89 ± 0.14[a]	2.41 ± 0.27[a,b]	2.49 ± 0.19[b]	4.72	2.15	0.026
RMR_4 (mL O_2 min^{-1})	3.70 ± 0.22	3.59 ± 1.15	3.63 ± 0.01	0.07	2.15	0.93
RMR_2 (mL O_2 min^{-1} gr^{-1})	2.11 ± 0.07	2.3 ± 0.06	2.32 ± 0.09	2.46	2.15	0.12
RMR_4 (mL O_2 min^{-1} gr^{-1})	2.14 ± 0.07	2.06 ± 0.1	1.95 ± 0.09	1.21	2.15	0.33
M_{sum2} (mL O_2 min^{-1})	5.86 ± 1.60[a]	12.68 ± 1.33[b]	13.54 ± 0.48[b]	8.44	2.10	0.007
M_{sum4} (mL O_2 min^{-1})	15.15 ± 0.47	8.62 ± 3.06	6.39 ± 1.67	5.02	2.3	0.11
M_{sum2} (mL O_2 min^{-1} gr^{-1})	4.12 ± 1.11	7.09 ± 0.19	7.24 ± 0.19	3.93	2.10	0.055
M_{sum4} (mL O_2 min^{-1} gr^{-1})	7.19 ± 0.04	4.14 ± 1.33	3.16 ± 0.78	5.55	2.3	0.098
ME (M_{sum2}/RMR_2)	3.04 ± 0.84	5.36 ± 0.47	5.48 ± 0.27	3.76	2.10	0.061
ME (M_{sum4}/RMR_4)	4.10 ± 0.12[a]	2.37 ± 0.09[b]	1.76 ± 0.45[b]	19.29	2.3	0.019

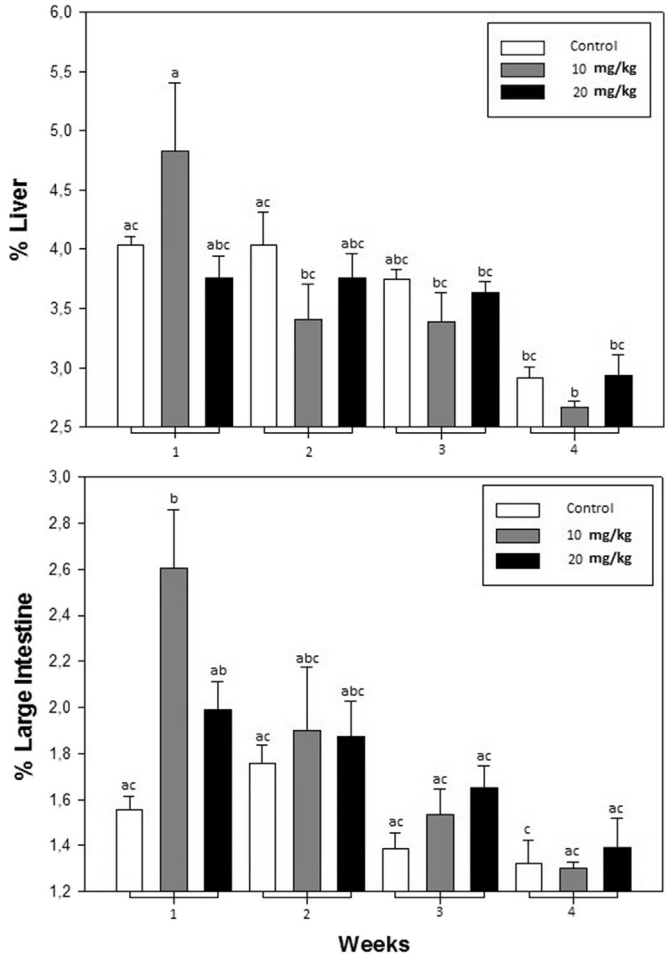

Fig. 1. Percentage of liver and large intestine mass related to total body mass (±SE) of *Coturnix c. japonica* exposed for four weeks to chlorpyrifos. Different letters denote significant differences ($p < 0.05$) after a *post hoc* Tukey test.

tion) revealed that in all cases significant differences along time were observed only for control birds (Figs. 2 and 3). The pesticide concentration caused a significant decrease in most of cases, with the exception of intestinal BChE, hepatic CbE-4NPA and hepatic BChE (Table 3). The *post hoc* Tukey test revealed that intestinal CbE-αNA activity was higher in the control group compared with both pesticide-treated groups (Fig. 2). Particularly, we found a 45% of inhibition in the fourth week of sampling. In the liver we also observed that control birds had significantly higher CbE-αNA activity than the pesticide-treated groups, but despite this tendency, no significant inhibitions were observed at any sampling time. In plasma, CbE-αNA activity was significantly depressed (54% inhibition of controls at the 4th week) by the highest chlorpyrifos dose (Fig. 2). Intestinal CbE-4NPA of the control group was significantly higher than that of pesticide-treated groups, although no significant inhibition was detected within weeks (Fig. 2). Although we observed a marginally significant effect of pesticide concentration on liver CbE-4NPA (see Table 3), the *post hoc* test revealed that 20 mg/kg treatment had lower values than the control group (Fig. 2). However, no significant differences were found between treatments within each week. In plasma, CbE-4NPA activity was significantly lower in the 20 mg/kg treatment than the control group with a 55% of inhibition in the fourth week (Fig. 2). We found no significant differences in BChE in both small intestine and liver, but a 66% inhibition in plasma during the fourth week in the 20 mg/kg treatment (Fig. 3). No significant effect of chlorpyrifos was observed on AChE activity in liver and small intestine

Table 3
Summary of two-way ANOVA for esterase activity of chlorpyrifos exposed quails.

Tissue	Esterase	Source of variation[a]	F	df	p
S.I.	CbE-4NPA	Time of exposure	6.31	3.36	<0.01*
		Concentration	4.56	2.36	0.017*
		Time of exposure* Concentration	0.84	6.36	0.55
	CbE-αNA	Time of exposure	3.19	3.36	0.035*
		Concentration	9.8	2.36	<0.01*
		Time of exposure* Concentration	1.6	6.36	0.17
	BChE	Time of exposure	2.98	3.36	0.04*
		Concentration	2.58	2.36	0.09
		Time of exposure* Concentration	0.57	6.36	0.75
	AChE	Time of exposure	4.75	3.36	<0.01*
		Concentration	0.12	2.36	0.87
		Time of exposure* Concentration	0.36	6.36	0.9
Liver	CbE-4NPA	Time of exposure	4.34	3.36	0.01*
		Concentration	3.07	2.36	0.058
		Time of exposure* Concentration	1.09	6.36	0.38
	CbE-αNA	Time of exposure	5.51	3.36	<0.01*
		Concentration	5.67	2.36	<0.01*
		Time of exposure* Concentration	0.37	6.36	0.89
	BChE	Time of exposure	1.15	3.36	0.35
		Concentration	2.2	2.36	0.12
		Time of exposure* Concentration	0.54	6.36	0.77
	AChE	Time of exposure	1.81	3.36	0.16
		Concentration	1.23	2.36	0.3
		Time of exposure* Concentration	0.82	6.36	0.56
Blood plasma	CbE-4NPA	Time of exposure	3.59	3.36	0.02*
		Concentration	8.02	2.36	<0.001*
		Time of exposure* Concentration	2.18	6.36	0.06
	CbE-αNA	Time of exposure	5.65	3.36	<0.01*
		Concentration	7.47	2.36	<0.01*
		Time of exposure* Concentration	1.88	6.36	0.1
	BChE	Time of exposure	3.08	3.36	0.035*
		Concentration	5.44	2.36	<0.01*
		Time of exposure* Concentration	2.49	6.36	0.034*

[a] Categorical independent variables: time of exposure = 1, 2, 3 and 4 weeks; treatment = 0, 10 and 20 mg/kg dry food.

(Table 3). Finally, after 30 days of exposure to chlorpyrifos, levels of glucuronic acid in the excreta exhibited a significant increase in the treatment with the highest pesticide concentration (ANOVA $F_{(2,13)} = 15.2$; $p = 0.0004$; Fig. 4).

4. Discussion

4.1. Chlorpyrifos effect on energy expenditure rates

Several studies have documented that ME exhibits some interspecific variability among passerine species, with values ranging from 3.3 to 9.0 (Liknes and Swanson, 1996; Arens and Cooper 2005). Values found here, fall well within that range. Most studies documenting variation in M_{sum} and resting metabolic rates in birds (resting and basal metabolic rates) have been performed as a function of natural and artificial changes in ambient temperature (e.g., Cooper and Swanson, 1994; Arens and Cooper 2005). In general, an increase of oxygen consumption can be attributed to a high thermogenesis associated with metabolic costs and cold tolerance during winter (McKechnie and Swanson, 2010). Nonetheless, there are few studies that assess the impact of other environmental stressors such as parasitism, on metabolic rates in endothermic species. In fact, some studies have shown that parasitism has a strong impact on the host energy budget (e.g., effect on RMR, daily expenditure and MMR). For instance, Careau et al. (2012) documented that infection of bot fly (*Cuterebra emasculator*) larvae on eastern chipmunks (*Tamias striatus*) increased the RMR and, in turn, decreased the M_{sum} and ME. Our results suggest that chlorpyrifos exposure generated an effect equivalent to that of parasitism on energy expenditure rates because of M_{sum} and ME decreased in the third and fourth week of both pesticide treatments.

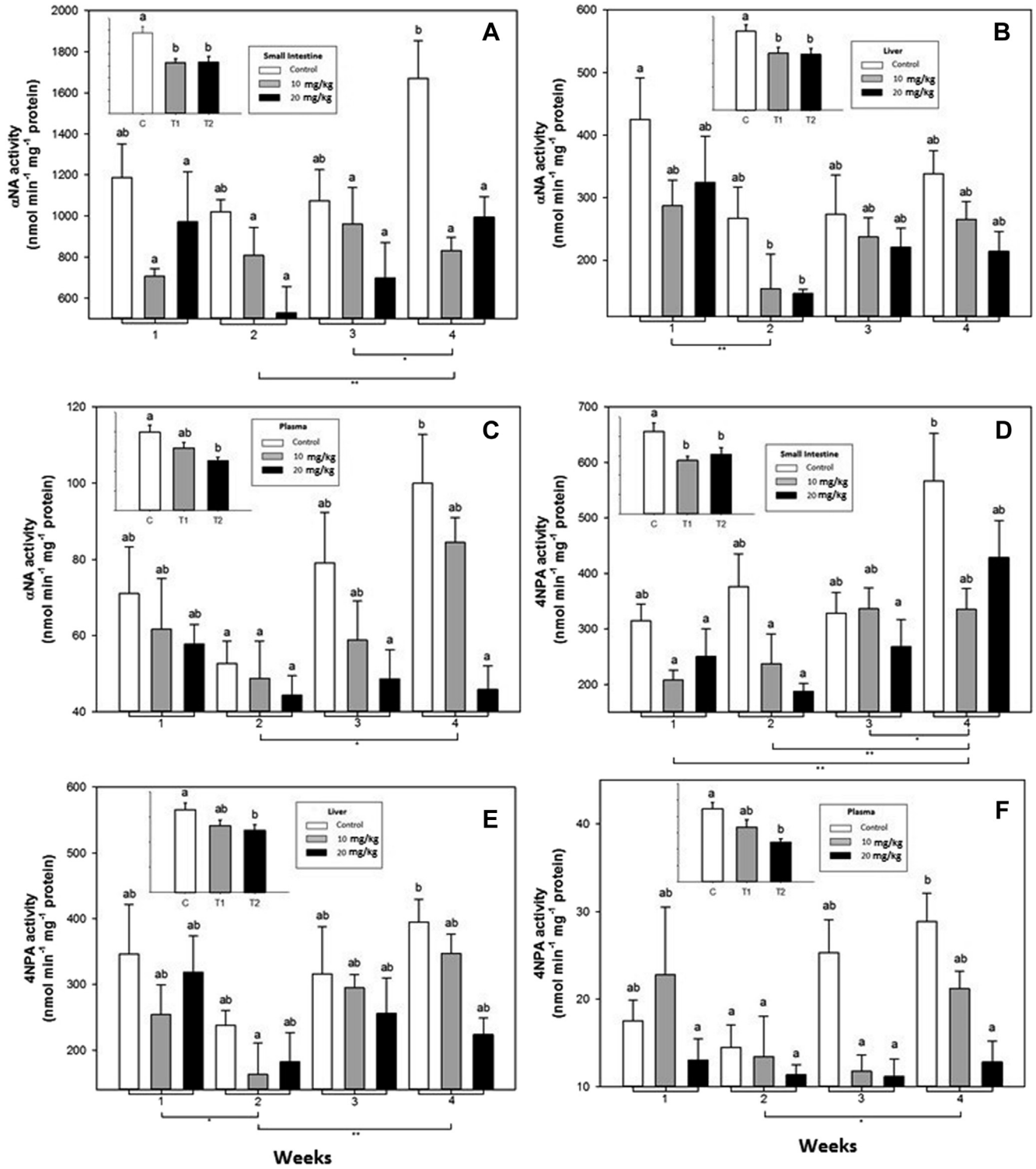

Fig. 2. Activity of esterases in different tissues (A: CbE-αNA of small intestine, B: CbE-αNA of liver, C: CbE-αNA of plasma, D: CbE-4NPA of small intestine, E: CbE-4NPA of liver, F: CbE-4NPA of plasma) of *Coturnix c. japonica* exposed for four weeks to chlorpyrifos. Inset small plots show the results of data pooled by treatment. Different letters denote significant differences ($p < 0.05$) after a *post hoc* Tukey test. Lines at bottom connecting groups denote significant differences between weeks (*$p < 0.05$, **$p < 0.01$).

According to the "barrel model" proposed by Weiner (1992), energy budgets are dynamic, and depend on the rates of energy input. Similarly, the resource allocation to the components of the energy budgets or energy outputs (e.g., maintenance, reproduction and growth) may be altered to allocate energy preferentially to some components at the expense of other, resulting in a trade-off among these components. For instance, in response to an increase in energy spent on activation of immune system, animals can reduce other components of its budget, such as maintenance, reproduction, growth and thermoregulation (Degen, 2006). Likewise, birds may sacrifice oxidative protection in response to increases in energy demands for reproduction (Wiersma

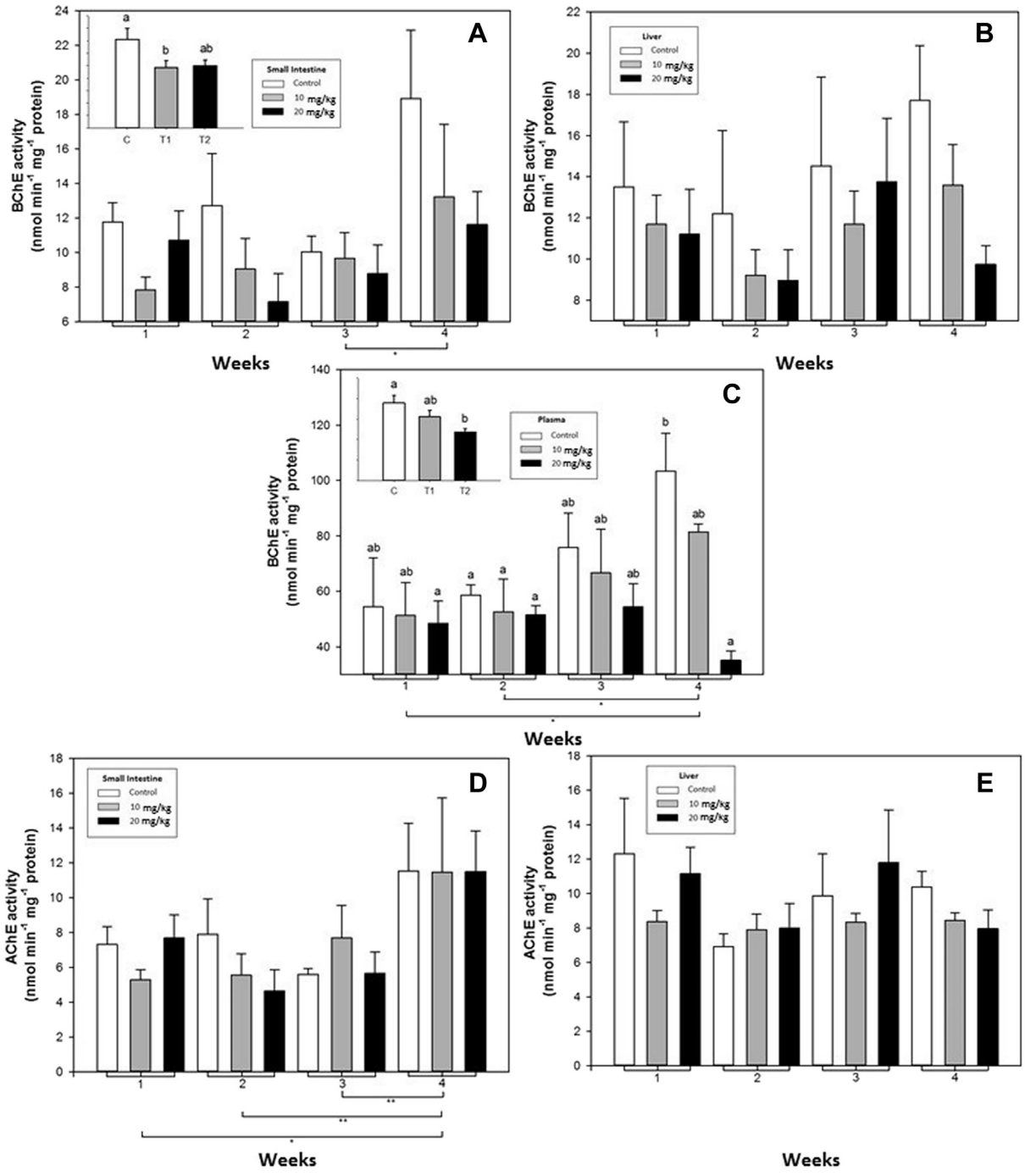

Fig. 3. Activity of esterases in different tissues (A: BChE in small intestine, B: BChE in liver, C: BChE in plasma, D: AChE in small intestine, E: AChE in liver) of *Coturnix c. japonica* exposed for four weeks to chlorpyrifos. Inset small plots show the results of data pooled by treatment. Different letters denote significant differences ($p < 0.05$) after a *post hoc* Tukey test. Lines at bottom connecting groups denote significant differences between weeks ($^{*}p < 0.05$, $^{**}p < 0.01$).

et al., 2004). Having low M_{sum} and ME values clearly is a disadvantage, because it is a combination of high maintenance costs and low thermogenic capacity. In this situation, it has been documented that parasitism (and the associated energy challenge) reduce survival in eastern chipmunks during a year of low food abundance (Careau et al., 2013). Moreover, pesticide exposure effects on the host's energy metabolism would be most apparent in animals with some energy challenge, as occurs in periods of low food availability or in juveniles that must cope with additional costs of growth (Careau et al., 2010). The exposure to OP pesticides could have adverse effects on species of birds during their reproductive stages, since egg production is costly and produces an increase in RMR (Vézina and Williams, 2005). Energy expenditure associated with egg production in Japanese quail is elevated. Ward and MacLoed (1992) observed an increase of 49% in daily energy expenditure in laying quails compared to individuals that have not yet started to lay eggs. We hypothesized that chlorpyrifos-exposed quails had a high metabolism maintenance costs, thereby sacrificing reproduction at the expense of a high detoxification rate.

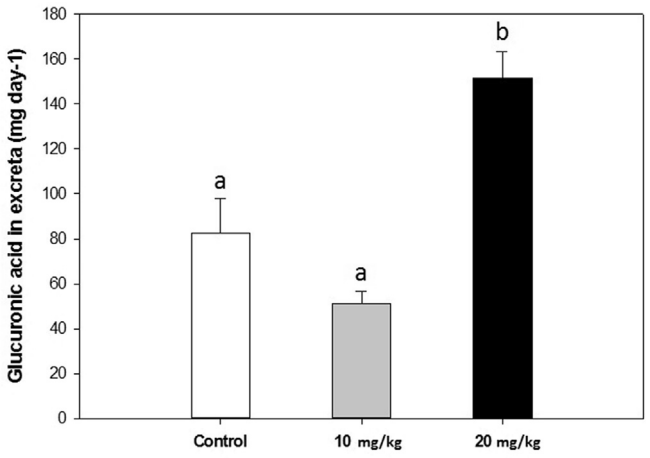

Fig. 4. Excretion rate of glucuronic acid in *Coturnix c. japonica* orally exposed to chlorpyrifos for four weeks. Bars are the mean values (±SE). Different letters denote significant differences ($p < 0.05$) after a *post hoc* Tukey test.

4.2. Chlorpyrifos effect on body mass and organ development

Changes in body mass of animals long-term exposed to OP pesticides have been previously reported. For instance, Nicolaus and Lee (1999) found that small amounts of OP affect the feeding behavior and body mass of breeding Red-winged Blackbirds (*Agelaius phoeniceus*). Besides, Grue and Shipley (1984) found a 14% average reduction in body mass of starlings following a sublethal exposure to the OP dicrotophos (see Young and Grandjean (1988) for an example in mammals). We found no significant differences in body mass of chlorpyrifos-exposed quails compared to non-treated birds. The low level of pesticide exposure in our study could explain differences between this and previous studies.

With regard to organ size, only liver and large intestine were affected by treatment, and an increase of the relative masses in the pesticide treatments compared with control was observed in large intestine (Fig. 1). Such increase in organs mass could suggest an initial effect of systemic toxicity that probably facilitates erythrocyte removal by the reticuloendothelial system (Mahmoud et al., 2012). This process has been described in aged erythrocytes, with no necrotic damage, that have suffered mechanical stress, energy depletion or an increase in oxidative processes that exceed antioxidant protection systems (Hermle et al., 2006; Quintanar-Escorza and Calderón-Salinas, 2006). It is noteworthy that in the study of Mahmoud et al. (2012), such results were found in liver, kidney and spleen of Japanese quails exposed to the OP pesticide malathion. Lack of significant differences in our study can be also attributed to the low level of chlorpyrifos exposure. While Mahmoud et al. used a dose corresponding to 20% of LD_{50}, we used a 7.3% and 15.7% of LC_{50} as described in Hill and Camardese (1986).

4.3. Detoxification of chlorpyrifos

In the present study, we found that in *Coturnix japonica*, BChE activities were inhibited by chlorpyrifos after pesticide exposure, whereas AChE activity was unaffected. AChE and BChE are thought to be the result of a gene duplication event early in vertebrate evolution (Pezzementi et al., 2011). Although, BChE and AChE are highly homologous proteins; these enzymes have distinct substrate preferences (Pezzementi et al., 2011; Fang et al., 2011). Another difference between these two cholinesterases is that AChE has two phenylalanine in its acyl pocket that is not present in BChE. Therefore, conformational differences in active sites between AChE and BChE; as well as substrate specificity linked to enantioselectivity of OP may account for the inhibitory effect of chlorpyrifos found only for BChE activity. Accordingly, many studies have shown that BChE activity is more sensitive to OP inhibition than AChE (Sanchez-Hernandez, 2001 and references therein).

Regardless of the OP exposure, our results revealed a trend towards an increase of esterase activities along the weeks in the Japanese quail. Although some data on esterase activity during organism growth are reported for other species, data for birds are scarce (Veini et al. 1986; Holmes and Masters, 1968; Tsitsiloni et al., 1993). These studies revealed that magnitude of change depend on the species and tissue analyzed. Although the mechanisms underlying this variation have not been fully clarified, the variation in the utilization of lipids during postnatal development is the most likely cause (Lakshmipathi and Sujatha, 1991). Our results suggest that in the case of Japanese quail this trend is particularly evident in untreated animals, because in those treated with pesticide, activity appears to be unchanged. Interestingly, this could be the inhibitory effect of chlorpyrifos on the enzymes present in tissues, although other mechanisms blocking enzyme expression should not be totally excluded (Casida and Quistad, 2005).

Inhibition of CbE and BChE activities by OP pesticides is a well-documented phenomenon in many organisms (Wheelock et al., 2008; Sanchez-Hernandez, 2001). In our study, however, there was no significant inhibition of esterase activity during the first two weeks following chlorpyrifos treatment. This absence of esterase inhibition may be explained by the high capacity of synthesis of new enzyme in birds. Avian brain AChE and plasma AChE and CbE activities recovers its activity, after OP inhibition, following a logarithmic pattern (Holmes and Boag, 1990). It has been suggested to occur in two steps: a rapid initial recovery that can be attributed to spontaneous enzyme reactivation and synthesis of new enzyme, and a slower second step returning to normal activity mainly due to *de novo* synthesis (Bartkowiak and Wilson, 1995). Because of the use of sublethal doses, during the first two weeks the detoxifying system in charge of esterases would work properly, but because it is a subchronic exposure, after that period this system would fail, resulting in inhibitions up to 66% (e.g., in plasma BChE). In parallel with these inhibitions, chlorpyrifos did not cause a significant effect on AChE activity. These results as a whole suggest that CbE and BChE are fulfilling their role as bioscavengers against pesticides, and therefore protecting the organism from the inhibition of AChE.

Glucuronic acid output increased when quails were exposed for 30 days to a 20 mg/kg dose of chlorpyrifos. It has been observed that the excretion of pesticide's metabolites by forming their conjugates constitute the chief mode of pesticides elimination in fishes, because conjugates are more readily excreted than lipophilic original pesticides (Katagi, 2010). In birds, species with greater diet breadth, and thus a greater input of plant secondary compounds, excrete a higher amount of glucuronic acid than strictly granivorous species after an intake of 8% of dietary phenolics (Ríos et al., 2012). Similar to that occurring with dietary phenolics metabolism, our results suggest that the glucuronidation pathway may be key for detoxify and eliminate ingested OP pesticides by birds. Notwithstanding, since to our knowledge, any study has related glucuronic acid in excreta with chlorpyrifos exposure in developing birds. Comparisons with previous results would be only speculative.

5. Conclusions

Exposure to sublethal doses of chlorpyrifos corresponding to 7–15% of LC_{50} in juvenile Japanese quails, caused an adverse effect on aerobic performance, eliciting a decrease in thermogenic capacity (lower M_{sum}). This effect caused a low metabolic expansibility, which probably leads to individuals with a lower capacity to toler-

ate colder temperatures. Current data showed that chlorpyrifos reduced the normal increase of esterase activity during quail growth, probably because an irreversible inhibition of both CbE and ChE activities by the OP. This finding means that esterases are fulfilling its buffering role, binding the pesticide and diminishing therefore its toxic capability. At the end of the experiment, we found a higher excretion rate of glucuronic acid in the excreta of quails exposed to the higher dose of chlorpyrifos. This result would corroborate a higher bioactivation of chlorpyrifos into chlorpyrifos-oxon in the 20-mg/kg group. These results suggest that, for a more detailed understanding of the toxic effects of pesticides on non-target organisms particularly in their juvenile phase, it is necessary to analyze more than one biomarker covering multiple levels of biological organization (e.g., enzymes and metabolic rate). In this sense, the use of blood enzyme activities facilitates this multibiomarker approach under a non-lethal context, which is of concern in endangered species.

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