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Comparative intestinal esterases amongst passerine species: Assessing vulnerability to toxic chemicals in a phylogenetically explicit context

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HIGHLIGHTS

- Passerine birds display interspecific differences in intestinal esterase and lipase.
- Phylogeny affected species-specific variations of esterase and lipase activities.
- Dietary nitrogen positively changed intestinal lipase activity.
- Field monitoring of organophosphorus exposure should consider bird phylogeny.

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ABSTRACT

Inhibition of blood esterase activities by organophosphate (OP) pesticides has been used as a sensitive biomarker in birds. Furthermore, compared to mammalian vertebrates, less is known about the role of these enzyme activities in the digestive tracts of non-mammalian vertebrates, as well as the environmental and biological stressors that contribute to their natural variation. To fill this gap, we examined butyrylcholinesterase (BChE) and carboxylesterases (CbE) in the digestive tracts of sixteen passerine species from central Chile. Whole intestine enzyme activities were positively and significantly correlated with body mass. After correcting for body mass and phylogenetic effect, we found only a marginal effect of dietary category on BChE activity, but a positive and significant association between the percentage of dietary nitrogen and the mass-corrected lipase activity. Our results suggest that observed differences may be due to the dietary composition in the case of lipases and BChE, and also we predict that all model species belonging to the same order will probably respond differently to pesticide exposure, in light of differences in the activity levels of esterase activities.

1. Introduction

Toxicity and metabolism of organophosphorus (OP) pesticides involve four esterase enzymes, i.e., acetylcholinesterases (EC 3.1.1.7), butyrylcholinesterases (EC 3.1.1.8), carboxylesterases (EC 3.1.1.1) and phosphotriesterases (EC 3.1.8.1). In the nervous tissue, inhibition of acetylcholinesterase (AChE) activity by OP compounds is the primary mechanism of toxic action of this class of agrochemicals (Fukuto, 1990), whereas the butyrylcholinesterase (BChE) and carboxylesterase (CbE) activities act as an efficient non-catalytic and stoichiometric mechanism of detoxification (Sogorb and Vilanova, 2002; Wheelock et al., 2008; Masson and Lockridge, 2010). The active site of these esterases is phosphorylated by the 'oxon' metabolite (oxygen analogue of phosphorothioate OPs) to yield a stable enzyme-inhibitor complex. Likewise, the oxon metabolites can be hydrolysed by the phosphotriesterases (Vilanova and Sogorb, 1999).

Over the last two decades, inhibition of blood cholinesterase and CbE activities by OPs has been used as a sensitive biomarker in both invertebrate (Guilhermino et al., 2000; Domingues et al., 2010; Malagnoux et al., 2014) and vertebrate species (Thompson and Walker, 1988; Sanchez et al., 1997; Soler et al., 1998; Nunes, 2011). The non-lethal nature of this approach together with the high sensitivity of blood esterases to OP inhibition have been the determining factors leading to the adoption of these biomarkers in birds (Thompson, 1991; Sogorb et al., 2007; Cheke et al.,

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2012). Many studies have identified the body size, age, sex and diet of birds as potential interfering sources in the interpretation of the levels of blood and liver CbE and BChE activities. For example, Bush et al. (1973) documented that birds with the greatest body mass had high levels of hepatic esterase activities compared to the smallest individuals. This was, however, questioned by Roy et al. (2005) and Sogorb et al. (2007), who found a negative correlation of plasma CbE activity with body mass in raptors. Likewise, diet is another potential factor of interspecies variation in blood esterase activity. It has been reported that omnivorous and herbivorous bird species have high blood CbE activity compared to insectivorous species (Bush et al., 1973; Roy et al., 2005; Thompson et al., 1988). In addition, recently Ríos et al. (2014) compared the CbE activity of the small intestine in two passerine species, finding that the higher activity in the omnivorous species would allow it to better deal with fats from different sources.

The main route of OP exposure in birds is believed to be through ingestion of contaminated food (Smith et al., 2007), although some recent studies have shown that acute exposure to organophosphorus pesticides by dermal or respiratory surfaces is also an important uptake route (Cheke et al., 2012). Therefore, the presence of pesticide-metabolizing esterases in the gastrointestinal epithelium would be the first biochemical barrier against pesticide uptake. However, as far as we know, no study has examined the role of these enzyme activities in the digestive tracts of non-mammalian vertebrates as well as the environmental and biological stressors contributing to their natural variation of activity. With the aim of describing and identifying the main biological factors contributing to inter- and intra-species differences in intestinal esterase activities and to assess putative toxicological impacts, we determined the levels of both BChE and CbE activity in the small intestines of sixteen passerine species inhabiting and foraging within the Chilean agroecosystem, covering a wide range of diet and size. Because lipase activity can interfere with the measurement of CbE activity when the latter is assayed with short-chain esters, this digestive enzyme was also included.

The main questions that we aimed to answer were: (1) Does the hydrolytic activity of small intestine esterases correlates with diet in passerines under a phylogenetically explicit context? (2) Is there any relationship between enzyme activity and body mass? (3) Does phylogeny impact the expression of these enzymes? It is our expectation that the present study will provide some answers to these questions, so more reliable assessments of pesticide exposure in field may be performed.

2. Materials and methods

2.1. Bird capture and tissue preparation

Birds were sampled in the following localities in Central Chile: Quebrada de la Plata (33°30′S, 70°54′W) and San Carlos de Apoquindo (33°23′S, 70°30′W), both of which are characterized by a Mediterranean climate. We captured a total of 114 adult individuals belonging to 16 species of songbirds using mist nets. To avoid the putative effect of dietary switches exhibited by some species in winter (Lopez-Calleja, 1995), captures were performed from November to January (austral summer). The captures were undertaken with the permission of the Chilean Agricultural Service (SAG).

Immediately after capture, the birds were transported to the laboratory, weighed, sacrificed by CO_2 exposure to be dissected and organs removed. The small intestine was rapidly excised, flushed with ice-cold saline solution (0.9% NaCl), measured (±0.1 cm) and weighed (±0.001 g) before being placed in storage at −50 °C. For biochemical assays, tissues were thawed and the whole small intestine was homogenized in 20 vol of 0.9% NaCl for 30 s at 24000 rpm using an Ultra Turrax T25 homogenizer (Janke and Kunkel, Breisgau, Germany). The homogenates were centrifuged at 5000 rpm for 10 min and 4 °C, and the supernatant was finally stored at −50 °C.

2.2. Esterase activities

Carboxylesterase activity was measured on an Asys HiTech UVM340 plate reader (Asys HiTech GmbH, Eugendorf, Austria) using the substrates alpha-naphthyl acetate (α-NA) and 4-nitrophenyl acetate (4-NPA) (Sigma-Aldrich, Madrid, Spain). The reason for using these two substrates was the existence of multiple CbE isoforms generally occurring in animal tissue with marked variability in substrate preference and sensitivity to OP inhibition (Wheelock et al., 2008). Hydrolysis of α-NA was measured as per the method described in Thompson (1999). Briefly, the enzymatic activity was run for 10 min at 40 °C in a reaction medium (200 μL, final volume) composed of 25 mM Tris–HCl (pH 7.6), 2 mM α-NA and the sample. The formation of α-naphthol was stopped by the addition of 75 μL of 2.5% SDS in 0.1% Fast Red ITR/2.5% Triton X-100, and the absorbance of the naphthol–Fast Red ITR complex was read at 530 nm after allowing the solution to stand for 30 min at 22–23 °C in the dark. The specific activity was calculated using a molar extinction coefficient of $33.2 \times 10^3\ M^{-1}\ cm^{-1}$ for the naphthol–Fast Red ITR complex. Hydrolysis of 4-NPA by CE (4-NPA-CE) was determined as described by Chanda et al. (1997). The reaction mixture (200 μL, final volume) contained 20 mM 4-NPA, 0.1 M Tris–HCl (pH 8.0) and the sample. The reaction was initiated by addition of 10 μL of 20 mM 4-NPA. The 4-nitrophenol liberated was read at 405 nm and quantified by a calibration curve (5–100 μM). Butyrylcholinesterase activity was determined according to Wheelock et al. (2005) as adapted from Ellman et al. (1961). The reaction medium (200 μL, final volume) was made up of 0.1 M Tris buffer (pH 8.0) containing 320 μM 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) and the sample. The reaction was initiated by addition of 60 mM butyrylthiocholine iodide (BTCI, Sigma-Aldrich, Madrid, Spain), and the product was monitored at 412 nm for 2 min. Specific activity was calculated using a molar absorption coefficient of $14.15 \times 10\text{-}3\ M^{-1}\ cm^{-1}$ (Eyer et al., 2003).

2.3. Lipase activity

Lipase activity was determined according to Gupta et al. (2002) using 4-NPP as the substrate. The reaction medium (250 μL, final volume) consisted of 50 mM Tris-HCl buffer (pH 8.0) containing 0.4% (w/v) Triton X-100 and the sample. The reaction was initiated with the addition of 20 μL of 16.5 mM 4-nitrophenyl palmitate (4-NPP) in dimethylsulfoxide, and the product (4-nitrophenol) was monitored at 412 nm for eight minutes. Specific activity was calculated using an external calibration curve made with 4-nitrophenol.

Protein concentration of each sample was determined by the Bradford method (Bradford, 1976) using bovine serum albumin as the standard. All enzyme activities were standardized by protein content of sample (IU mg^{-1} protein) and by gram of wet tissue (IU g^{-1} wet tissue). We also calculated the summed activity for each enzyme by using the total weight of the small intestine, and data are presented as total hydrolytic activity (IU min^{-1}; 1 IU = 1 μmol of substrate hydrolyzed per min under the experimental conditions in this study). Although it is usual to measure the activities of membrane-bound enzymes in isolated preparations (Martinez del Rio and Stevens, 1989), we used homogenates of intestinal tissue, which yield consistent data of the activity of brush-border enzymes (Martinez del Rio et al., 1995; Brzek et al., 2013). In addition, the inefficient recovery of enzymes from brush-border isolated vesicles (Vasseur, 1989), may result in an

underestimation of enzymatic capacity. Thus, the homogenate likely gives a good indication of brush-border membrane activity that can be compared among species of birds (Ramirez-Otarola et al., 2011).

2.4. Phylogeny analysis

We used a phylogenetic tree based on Sibley and Alquist's (1990) hypothesis (Fig. 1). To maintain consistency with our earlier work (Sabat et al., 2010; Ramirez-Otarola et al., 2011), we used the original backbone proposed by Sibley and Ahlquist (1990) despite the existence of more recent avian phylogenies (Fain and Houde, 2004; Barker et al., 2004; Hackett et al., 2009). Although some species were not listed, we used other species belonging to the same genus to determine branch lengths in our phylogeny. However, it was not possible to use a congeneric species with some species, thus we used data from other phylogenies to establish the branch length. As suggested by Swanson and Garland (2009), we extrapolated arbitrary branch lengths between *Asthenes humicola* and *Leptasthenura aegithaloides* based on the data of Gonzalez and Wink (2008), whereas the phylogeny of the Family Tyrannidae was obtained from Ohlson et al. (2007). The phylogenetic distance between *Sturnella loyca* and *Curaeus curaeus* was derived from the phylogeny proposed by Lanyon and Omland (1999).

2.5. Data analysis

For an exploratory purpose, we first tested for interspecific differences in enzyme activity expressed as IU g^{-1} of wet tissue. In this analysis, we removed from consideration the species *Xolmis pyrope*, one of the 16 species, because we only obtained data from one individual. To assess the possible effect of phylogenetic inertia on enzymatic activity (Blomberg et al., 2003), we calculated the *K*-statistic using the *Independent Contrasts* (v2.18) software (P. Withers, personal communication). The *K*-statistic reflects the degree of similarity among relatives, assuming a Brownian motion model and a specified species' phylogeny (topology and branch length). This statistical probe tests the null hypothesis of no pattern of similarity among relatives. A $K = 1$ means that a trait has the expected amount of phylogenetic signal. Values of $K < 1$ mean that closely related species are more different from each other than expected, and $K > 1$ indicate that closely related species are more similar than expected (see Blomberg et al., 2003 and Ackerly, 2009). To remove the phylogenetic inertia effects, we used Phylogenetic Independent Contrast (PIC) and Phylogenetic Autocorrelation (PA) using *Independent Contrasts* software (v 2.18).

We tested for a functional association between body mass and enzymatic activity, performing both conventional least squares regression (CLSR) and regression on phylogenetic independent contrasts (PIC, Felsenstein, 1985). Because the three enzyme activities were positively correlated with body mass, we corrected for this by calculating the residuals from least squares linear regressions both the conventional way and through the origin with phylogenetic independent contrasts (PICs, see Garland et al., 1992; Cheverud et al., 1985; Rohlf, 2001). With the purpose of testing the impact of dietary habits and composition on enzymatic activities, we examined the possible relationships between mass-independent enzymatic activities (i.e., the residuals of regression of total enzyme activity against body mass and mass of small intestine) and the percentage of nitrogen, as well as the percentage of prey items in the gut content of each species, using conventional

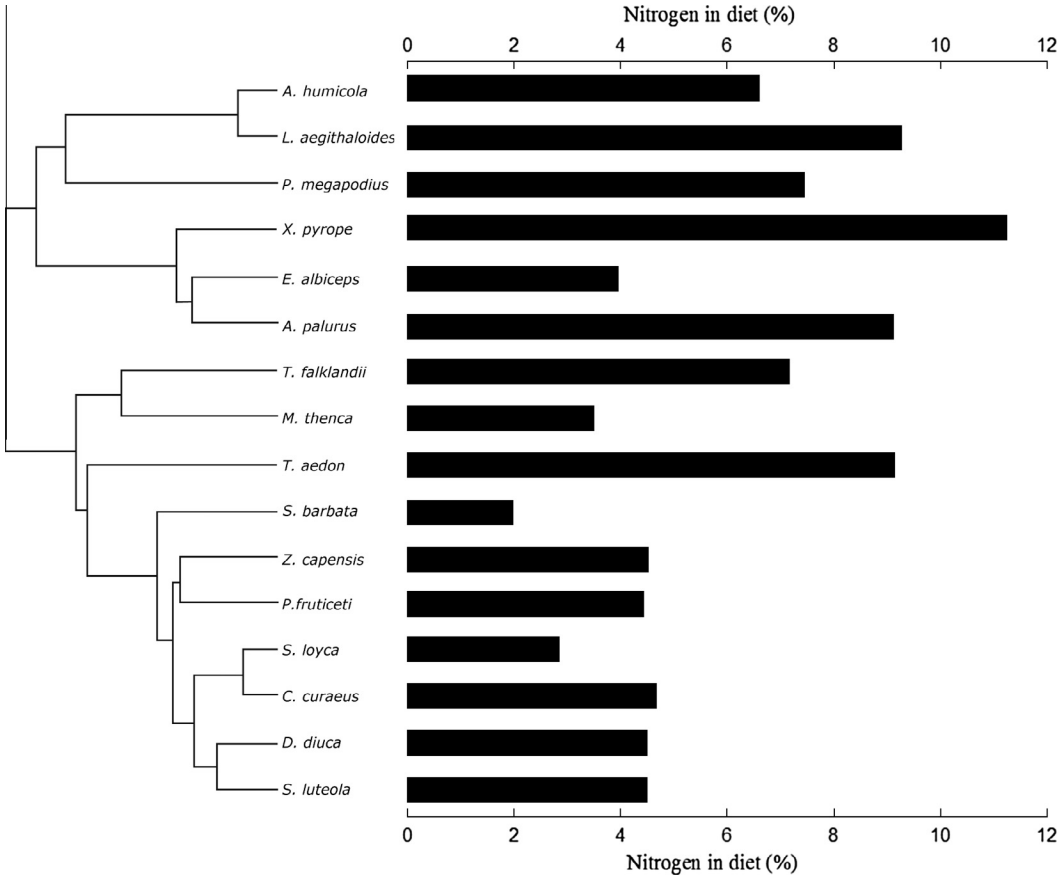

Fig. 1. Phylogenetic tree of the passerine birds used in this study. Dietary nitrogen percentages are shown as bars. Modified from Ramirez-Otarola et al. (2011).

correlations and correlations on phylogenetic independent contrasts (Felsenstein, 1985). The rationale of the independent contrasts is to use phylogenetic information to transform the n original, non-independent species values into n–1 independent distributed contrasts (Garland et al., 2005). These contrasts are constructed computing (weighted) differences ("contrasts") between the character values of pairs of sister species and/or nodes, as indicated by a phylogenetic topology. Usually, contrasts are standardized by the square root of the expected variance, which is proportional to branch length. Such contrasts from biochemical (e.g., enzyme activities) and ecological relevant data (e.g., nitrogen content in diet) can then be used in conventional correlations to infer functional association among the original variables. Finally, a Kruskal–Wallis ANOVA was used to test if the dietary category affects the activity of the intestinal enzymes, taking into account the phylogenetic residuals obtained in the PA analysis. We used dietary categories from the literature, which assigned the bird species as granivores, insectivores and omnivores. The numbers of cases for each variable may differ because some tissues were lost and we were unable to obtain measurements of different enzymes in all birds.

3. Results

3.1. Interspecific differences

Table 1 summarizes the arithmetic mean (±1 SD) of esterase and lipase specific activities recorded in the small intestines of the birds, together with their body mass and dietary habits. We found strong interspecific differences in the model species for specific activities activities standardized by gram of wet tissue (CbE-αNA: $F_{(14,95)} = 5.73$, $p < 0.001$; CbE-4NPA: $F_{(14,99)} = 2.42$, $p = 0.006$; BChE: $F_{(14,76)} = 11.9$, $p < 0.001$; lipase: $F_{(14,94)} = 5.36$, $p < 0.001$) and by protein content of the sample (CbE-αNA: $F_{(14,95)} = 5.59$, $p < 0.001$; CbE-4NPA: $F_{(14,99)} = 3.63$, $p < 0.0001$; BChE: $F_{(14,76)} = 8{,}37$, $p < 0.001$; lipase: $F_{(14,94)} = 3.4$, $p = 0.0002$). For both standardizations, we observed the highest values of CbE (α-NA) in the granivore *Diuca diuca* and the lowest in the insectivore *Troglodytes aedon*. For CbE (4-NPA), we only found differences between the omnivore *Zonotrichia capensis* and *T. aedon*. For BChE activity, we recorded the highest values in the granivores *Sicalis luteola* and *Z. capensis* and the lowest in the omnivores *Turdus falcklandii*, *S. loyca* and *C. curaeus*. In this case, BChE standardized by mg protein was also lower in the insectivore *A. humicola.* Finally, we found the highest values of lipase activity in the insectivore *Anairetes parulus*.

3.2. Phylogenetic signal

The activities of a-NA and 4-NPA showed significant phylogenetic signals based on 1000 randomizations ($k = 0.57$; $p < 0.001$ and $k = 0.46$; $p = 0.001$, respectively). The phylogenetic signal of BChE activity was also significant ($k = 0.83$; $p = 0.03$). Finally, lipase activity showed a significant phylogenetic signal ($k = 0.45$; $p < 0.03$). Thus, the presence of a significant phylogenetic signal among all traits justified the use of a phylogenetically based statistical method.

Table 1
Dietary habits, body mass and intestinal hydrolase activities of passerines birds from Chile.

Species	Family	*N*	Diet[a]	Mb (g)	CbE (α-NA) [b]	CbE (4-NPA)[b]	BChE [b]	Lipase[b]
Phrygilus fruticeti	EMB	3	S	39.1 ± 0.6	35.03 ± 6.06^{a,b,c,d} (4.91 ± 1.32)	24.54 ± 3.1^{a,b} (3.86 ± 1.04)	5.88 ± 1,79^{a,b,c} (859.43 ± 77.82)	0.14 ± 0.07^{a,b} (28.21 ± 20.64)
Sporagra barbata	FRI	9	S	14.3 ± 0.7	16.3 ± 5.47^{c,d} (2.38 ± 0.56)	8.1 ± 2.36^{a,b} (1.06 ± 0.22)	1.02 ± 0.1^{e} (135.97 ± 20.83)	0.049 ± 0.01^{b} (7.52 ± 1.78)
Diuca diuca	EMB	6	S	33.7 ± 0.6	82.49 ± 15.38^{a} (7.67 ± 1.88)	15.06 ± 2.7 ^{a,b} (1.37 ± 0.26)	4.07 ± 0.96 ^{a,b,c} (349.22 ± 60.56)	0.15 ± 0.06^{b} (13.99 ± 2.47)
Sicalis luteola	EMB	9	S	15.4 ± 0.3	27.31 ± 60^{c,d} (2.98 ± 0.68)	10.07 ± 1.36 ^{a,b} (1.05 ± 0.13)	7.12 ± 1.33^{a} (791.28 ± 160.5)	0.11 ± 0.028^{b} (11.63 ± 3.01)
Zonotrichia capensis	EMB	7	S/I	20.7 ± 0.6	65.9 ± 9.92^{a,b} (6.42 ± 0.82)	20.16 ± 2.11^{a} (1.94 ± 0.14)	5.35 ± 0.91^{a,b} (561.83 ± 138.9)	0.092 ± 0.015 ^{b} (8.54 ± 0.78)
Turdus falcklandii	TUR	10	S/I/F	78.8 ± 2.4	35.16 ± 4.43^{b,c,d} (3.3 ± 0.38)	8.4 ± 1.08 ^{a,b} (0.78 ± 0.1)	0.4 ± 0.08^{e} (36.62 ± 6.15)	0.069 ± 0.009^{b} (6.37 ± 0.57)
Mimus thenca	MIM	10	S/I/F	65.9 ± 2.3	27.28 ± 5.10^{c,d} (2.75 ± 0.33)	8.81 ± 1.74 ^{a,b} (0.9 ± 0.14)	2.18 ± 0.42^{c,d,e} (278.33 ± 58.82)	0.051 ± 0.015^{b} (6.44 ± 1.82)
Curaeus curaeus	ICT	10	S/I	89.6 ± 3.2	20.69 ± 4.93^{c,d} (2.24 ± 0.54)	9.42 ± 2.03 ^{a,b} (1.17 ± 0.3)	0.66 ± 0.11^{e} (93.01 ± 27.29)	0.14 ± 0.045^{b} (22.61 ± 11.63)
Sturnella loyca	ICT	9	S/I	88.5 ± 3.6	20.8 ± 3.71^{c,d} (2.22 ± 0.33)	10.2 ± 1.6^{a,b} (1.18 ± 0.18)	0.6 ± 0.18^{e} (71.23 ± 20.73)	0.056 ± 0.01^{b} (7.56 ± 1.73)
Pteroptochos megapodius	RHY	2	S/I	140.6 ± 5.2	56.84 ± 35.71^{a,b,c,d} (5.03 ± 0.3)	26.88 ± 8.55 ^{a,b} (4.3 ± 0.2)	0.64 (150.18)	0.027 ± 0.01^{b} (6.25 ± 0.5)
Elaenia albiceps	TYR	10	I/F	13.6 ± 0.5	23.88 ± 7.12^{c,d} (4.12 ± 1.16)	16.59 ± 6.09^{a,b} (1.77 ± 0.54)	1.02 ± 0.21^{d,e} (183.65 ± 47.48)	0.044 ± 0.0067 ^{b} (10.13 ± 2.34)
Xolmis pyrope	TYR	1	I	32.7 ± 0.9	23.32 (1.29)	25.7 (1.43)	– –	0.13 (7.22)
Asthenes humicola	FUR	7	I	21.4 ± 0.3	34.65 ± 14.87^{b,c,d} (2.28 ± 0.44)	14.44 ± 5.82 ^{a,b} (1.03 ± 0.45)	0.78 ± 0.23^{d,e} (73.5 ± 11.18)	0.088 ± 0.023^{b} (10.53 ± 3.71)
Anairetes parulus	TYR	5	I	5.5 ± 0.2	12.04 ± 3.12^{c,d} (1.82 ± 0.36)	6.68 ± 2.08 ^{a,b} (0.77 ± 0.24)	0.65 ± 0.087^{d,e} (97.63 ± 6.6)	0.31 ± 0.071^{a} (54.29 ± 18.16)
Troglodytes aedon	TRO	12	I	8.96 ± 0.26	8.04 ± 1.81^{d} (1.03 ± 0.26)	5.98 ± 1.77^{b} (0.83 ± 0.29)	1.97 ± 0.19^{c,d,e} (276.94 ± 48.04)	0.059 ± 0.0073^{b} (8.3 ± 1.39)
Leptasthenura aegithaloides	FUR	4	I	7.8 ± 0.32	55.99 ± 21.78^{a,b,c} (6.19 ± 2.36)	10.29 ± 3.5^{a,b} (1.32 ± 0.57)	1.36 ± 0.69^{b,c,d,e} (215.72 ± 83.21)	0.11 ± 0.028^{b} (18.45 ± 4.52)

Values in brackets expressed as IU mg^{-1} proteins (CbE-αNA and CbE-4NPA activity) and IU g^{-1} proteins (BChE and lipase activity); Letters denote significant differences (<0.05) after a Tukey test. EMB Emberizidae, FRI Fringillidae, FUR Furnaridae, ICT Icteridae, MIM Mimidae, RHY Rhynocriptidae, TRO Troglodytidae, TUR Turdidae, TYR Tyranidae.

[a] S = seeds; I = insects; F = fruits.

[b] Enzyme activity expressed as IU g^{-1} tissue.

3.3. Effect of body mass on enzyme activities

All whole intestine enzyme activities were positively and significantly correlated with body mass (Fig. 2). The allometric equations for each enzyme were: CbE-αNA = $0.35mb^{1.22\pm0.18}$ ($r = 0.76$, $p < 0.0001$); CbE-4NPA = $0.17mb^{1.17\pm0.16}$ ($r = 0.82$, $p < 0.0001$); BChE = $0.07mb^{0.8\pm0.29}$ ($r = 0.38$, $p = 0.02$) and lipase = $0.004mb^{0.8\pm0.19}$ ($r = 0.56$, $p < 0.001$). The phylogenetic analysis was consistent with the results obtained with conventional statistical regressions. The models described by the regressions based on PIC were: CbE-αNA activity = $-0.01mb^{1.17}$ ($r = 0.68$, $p < 0.0001$); CbE-4NPA activity = $-0.02mb^{1.3}$ ($r = 0.82$, $p < 0.0001$); BChE activity = $0.03mb^{0.63}$ ($r = 0.30$, $p = 0.04$) and lipase activity = $0.04mb^{0.93}$ ($r = 0.53$, $p = 0.002$). In most cases, the slopes of the allometric equations changed slightly. The exponents of CbE-αNA, CbE-4NPA, BChE and lipase activities obtained from the allometric equations differed significantly from 1 (t test: $p = 0.0002$, $p = 0.0007$, $p = 0.02$, and $p = 0.0008$ respectively). Moreover, none of the studied enzymes expressed as UI/g wet tissue were related with body mass (CbE-αNA, $r = 0.33$, $p = 0.21$; CbE-4NPA, $r = 0.46$, $p = 0.19$; BChE, $r = 0.24$, $p = 0.38$; and lipase, $r = 0.16$, $p = 0.63$). The phylogenetic analysis was slightly different in comparison to the results obtained with conventional regressions, because the specific activities of CbE-4NPA were positively correlated with body mass (CbE-αNA, $r = 0.40$, $p = 0.12$; CbE-4NPA, $r = 0.60$, $p = 0.01$; BChE, $r = 0.07$, $p = 0.80$; and lipase, $r = 0.35$, $p = 0.19$).

3.4. Effect of diet on enzyme activities

We analyzed the effect of diet on esterase and lipase activities using two approaches, i.e., dietary category and specific compounds in the diet. Dietary category (insectivore, frugivore and granivore) had no significant effect either on CbE-αNA, CbE 4-NPA or lipase activities (Kruskal–Wallis ANOVA test on mass independent enzymatic activity: $H_{(2,16)} = 2.46$, $p = 0.29$; $H_{(2,16)} = 3.61$, $p = 0.16$; $H_{(2,16)} = 3.75$, and $p = 0.15$ respectively). We found only a marginally significant effect of dietary category on BChE activity (Kruskal–Wallis ANOVA on mass independent enzymatic activity: $H_{(2,15)} = 5.74$, $p = 0.054$). In this case, BChE activity appears to be higher in granivores than in omnivores and insectivores. Furthermore, the Kruskal–Wallis ANOVA test on specific activities revealed that dietary category had no effect on any of the studied enzymes, although a tendency to increase the activity of lipase in insectivores was found for activities expressed by gram of wet tissue (CbE-αNA: $H_{(2,16)} = 2.02$, $p = 0.36$; CbE 4-NPA: $H_{(2,16)} = 3.06$, $p = 0.36$; BChE: $H_{(2,15)} = 3.07$, $p = 0.21$; lipase: $H_{(2,16)} = 5.17$, $p = 0.07$ for IU/g tissue and CbE-αNA: $H_{(2,16)} = 4.05$, $p = 0.11$; CbE 4-NPA: $H_{(2,16)} = 2.0$, $p = 0.37$; BChE: $H_{(2,15)} = 2.62$, $p = 0.27$; lipase: $H_{(2,16)} = 2.71$, $p = 0.26$ for activities expressed as IU/mg protein).

For all enzymes, it was observed that the percentage of dietary nitrogen, insects or seeds in the food did not correlate with the residuals of the conventional regression analyses using body mass

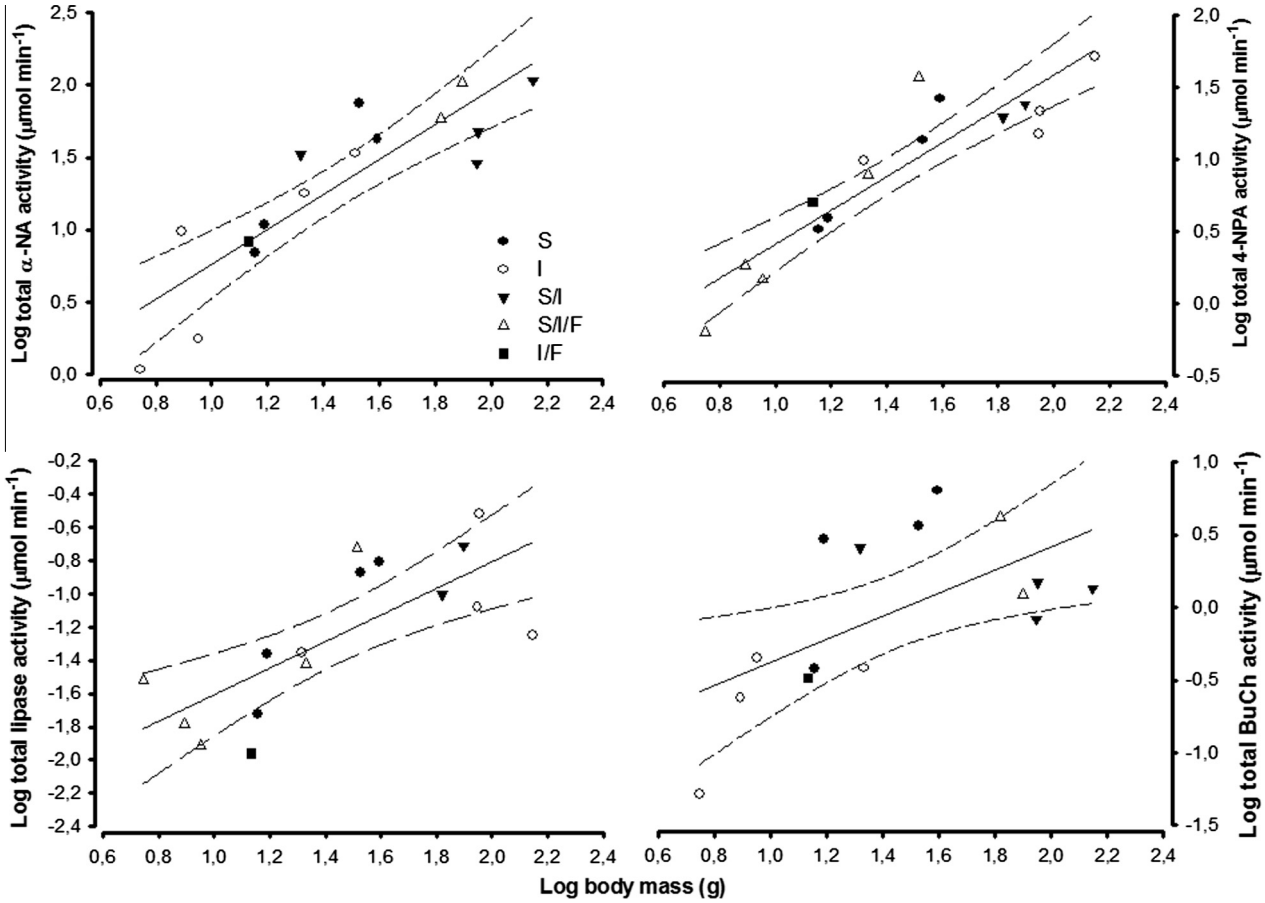

Fig. 2. Total enzyme activity against with body mass. The enzymatic activity of the three enzymes analyzed increased significantly with body mass. Panels show the allometric relationships for total CbE, BuCh and lipase activities. Symbols denote species food habits (S seeds, I insects, F fruits). 95% confidence intervals are shown as dashed lines.

Table 2
Correlations of residuals obtained after plotting enzymatic activity against body mass and mass of small intestine with dietary variables for 16 species of passerine birds, using conventional and phylogenetically (PIC) corrected analysis.

Residuals	Diet	Residual from intestine mass				Residual from body mass			
		CLSR		PIC		CLSR		PIC	
		r	*p*	*r*	*p*	*r*	*p*	*r*	*p*
α-NA	Seeds	0.19	0.46	0.32	0.24	0.26	0.31	0.053	0.86
	Insects	−0.17	0.51	−030	0.27	−0.27	0.32	−0.058	0.85
	Dietary N	0.05	0.86	0.02	0.95	−0.02	0.95	0.18	0.55
4-NPA	Seeds	−0.24	0.37	−0.23	0.41	−0.25	0.34	−0.015	0.96
	Insects	0.24	0.36	0.23	0.40	0.25	0.35	0.013	0.97
	Dietary N	0.05	0.46	−0.31	0.26	0.21	0.46	−0.15	0.60
BChE	Seeds	0.11	0.56	−0.09	0.76	0.19	0.53	−0.075	0.80
	Insects	−0.11	0.55	0.09	0.76	−0.19	0.52	0.02	0.95
	Dietary N	−0.39	0.04	−0.17	0.57	−0.38	0.19	−0.032	0.91
Lipase	Seeds	−0.30	0.25	−0.64	0.01	−0.07	0.80	−0.51	0.06
	Insects	0.29	0.26	0.63	0.01	0.05	0.84	0.48	0.08
	Dietary N	0.08	0.77	0.48	0.068	0.21	0.43	0.65	0.01

as the covariable (Table 2). Additionally, the correlations on phylogenetic independent contrasts revealed a similar pattern as observed with conventional analysis for carboxylesterases and butyrylcholinesterase (Table 2). However, when we removed the phylogenetic effect, a positive and significant association between the percentage of dietary nitrogen and the residuals of lipase activity was detected (Table 2, Fig. 3). The results of the total enzymatic activity corrected for small intestine mass yielded slightly different results. The CLSR revealed that the residuals of total BChE activity was positively correlated with nitrogen in diet (Table 2). Besides, the PIC analyses revealed a significant and positive association between the percentage of insects and seeds and lipase activity (Table 2).

4. Discussion

It is well known that differences in the digestive enzyme activities amongst bird species may be genetically based and also modulated by dietary substrates, a phenomenon known as physiological flexibility (Sabat et al., 1998; Ramirez-Otarola et al., 2011; Brzek et al., 2013). However, the modulation of digestive enzyme activity by specific chemicals in food is not a generalized phenomenon. For instance, comparative studies have reported that intestinal carbohydrases, but not proteases, are matched in an evolutionary sense to the nitrogen and carbohydrate content of natural diets in passerines (Ramirez-Otarola et al., 2011; Kohl et al., 2011). By comparing the reported experimental response to artificial diets and the observed differences in the field, Ramirez-Otarola et al. (2011) suggested that differences in disaccharidase activities among passerine species are probably the result of genetic differences amongst species brought about by natural selection. Continuing in this line, recently Ríos et al. (2014) reported that CbE and BChE activities in two passerine species (*Z. capensis* and *D. diuca*) remained unchanged after a month of dietary acclimation to diets experimentally enriched with unsaturated and saturated fatty acids, but both enzyme activities did however vary with season. The findings by Ríos et al. (2014) suggest, therefore, that interspecific differences in enzyme activities may be caused by a combination of phenotypic plasticity and genetic constraints.

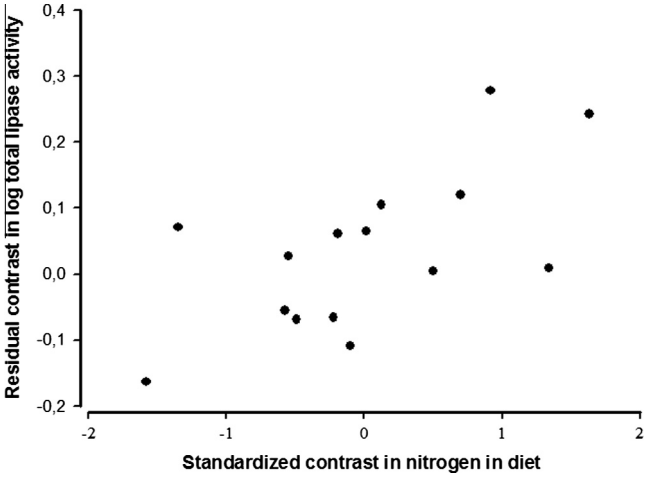

Fig. 3. Residuals contrast of total lipase activity against contrast of body mass as a function of the contrast of the percentage of dietary nitrogen.

In our study, summed intestinal esterase and lipase activities increased with body mass. The allometric exponents for BChE and lipase activities were similar to those previously reported for the intestinal sucrase and maltase activities in passerine birds (Shondube and Martinez del Rio, 2004). Nevertheless, the allometric exponents for CbE activity (both substrates) were slightly greater than those for the BChE and lipase activities. Given that our findings are based on a limited number of birds with a relatively small size, the results could reflect an overestimation of the allometric exponent. However, specific activities were weakly and non-significantly correlated with body mass, except for the hydrolysis of 4-NPA, which was positively correlated with body mass once the phylogenetic effect was removed. Body mass is a well-known trait that contributes to inter-species differences in ChE and CbE activities. For instance, Roy et al. (2005) found that plasma CbE activity towards α-NA of twenty European raptor species changed inversely with size. The same tendency was also observed by Sogorb et al. (2007) in eleven bird species, and by Lajmanovich et al. (2008) in amphibians. However, our results do not support these previous observations. The reason for this discrepancy is not clear, although a plausible explanation could be attributed to tissue-specific differences in esterase activity.

Our findings suggest that when phylogenetic effect and body mass were explicitly removed, dietary categories had little or no effect on both CbE and BChE activities in our model species. Only BChE activity was higher in granivores than omnivores and insectivores, but just a marginal significance was found. This trend, however, is supported by the higher activity of this enzyme in the granivores *S. luteola* and *Z. capensis* using conventional analysis and by the negative association we found between dietary nitrogen and the residuals of total BChE against mass of small intestine.

These findings suggest that gut BChE activity could display a similar trend with diet as that for blood esterase activities (Bush et al., 1973; Thompson, 1991, 1993). These authors suggested that the role of blood and liver CbE activity in the metabolism of lipophilic esters might explain this relationship. Thus, birds feeding on seeds or fruits should have higher levels of CbE activity because of higher concentrations of plant secondary metabolites which could act as inducers of CbE activity. Conversely, carnivorous species would have low pesticide-metabolizing enzymes such as CbEs and their detoxification capacity would be in their prey. However, some reservation should taking into account because differences in tissue expression of this esterase. Indeed, Cheke et al. (2012) measured blood cholinesterase activities of a wide range of East African birds, and no apparent relationship was evident between blood BChE activity and some traits such as sex or body mass. Beside tissue-related differences in esterase activity, phylogenetic origin may help to data interpretation in an ecological context (see Garland et al., 1992).

It is also possible that broad dietary categories, such as the ones we have used here may not capture the potential variation in the diet of a species and also do not incorporate the variations in the specific macromolecular compounds in foods (Cruz-Neto and Bozinovic, 2004). Such potentially confounding effects on physiological features in animals can be only be determined by analysing the specific compounds, such as the kind of dietary fatty acids, or other nutrients present in diets (Sabat et al., 2010). Remarkably, when we tested for a possible link between specific compounds in the diet, we found that the residuals of lipase activity were positively and significantly correlated with the percentage of nitrogen (Fig. 3), and also (although only marginally significant) with insect content. Similarly, the residuals of lipase activity against the mass of the intestine were positively and significantly correlated with the percentage of insects in diet. Surprisingly, this correlation was only found if the impact of phylogenetic relationships is removed. This pattern agrees with the tendency to find higher lipase activities in insectivores when we analysed specific activities on PA data and the highest value found in the insectivore *A. parulus* (Table 1). Because dietary nitrogen content is highly correlated with the percentage of insects consumed (Ramirez-Otarola et al., 2011), this relationship between lipase activity and dietary nitrogen could be explained by the fact that insectivorous species ingest a greater lipid content in their diets than frugivores and omnivores, who consume carbohydrate-rich pulps but with very low lipid content (Jordano, 1992). In addition, it has been reported that in vertebrates, many lipases participate in the digestion, absorption and reconstitution of fat and lipoprotein metabolism (Desnuelle, 1986). It has been also observed that in some cases, lipases catalyse a potentially useful series of reactions involved in protein metabolism, and even in the synthesis of peptides (Margolin and Klibanov, 1987; Langrand et al., 1988; West and Wong, 1987; Valivety et al., 1992). These functions of lipases, which have been documented in other vertebrate species, could contribute towards an explanation of the association with nitrogen intake in our study.

Finally, to our knowledge, this is the first report of the phylogenetic signal of intestinal esterase and lipase activities in birds. Even though the phylogenetic signal of the three enzymes was significant, all values were less than the unity, indicating that closely related species are more different from each other than expected for its phylogenetic origin. The current results agreed with previous works that assessed the phylogenetically-related differences in the membrane-bound intestinal enzymes sucrase, maltase and aminopeptidase-N (Ramirez-Otarola et al., 2011), as well as other physiological traits of birds (Blomberg et al., 2003). We encourage using phylogenetically oriented studies in order to assess vulnerability to toxic chemicals, especially when the species concerned are phylogenetically distant.

5. Conclusions

In summary, current data show that passerine species inhabiting agricultural areas in Chile display wide and interspecific differences in intestinal esterase and lipase activities. Inter-specific differences are due in part to dietary composition as in the case of lipases and probably for BChE activity, highlighting the importance of considering multiple biomarkers of wildlife exposure to OP pesticides. In turn, our study reveals that, despite all species in our experiment belonging to the same order, they will probably respond differently to pesticide exposure, in light of huge differences observed in the activity levels of esterases. Furthermore, our data provide baseline levels of pesticide-detoxifying esterase activities for a wide range of passerine species inhabiting agricultural areas, which may be used for field monitoring surveys following pesticide applications.

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