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The use of a lacertid lizard as a model for reptile ecotoxicology studies: Part 2 – Biomarkers of exposure and toxicity among pesticide exposed lizards

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ABSTRACT

As part of a wider study examining the impacts of corn pesticides on lacertid lizards in north-western Portugal, we examined various physiological, biochemical, and histological biomarkers of exposure and effect among field populations of *Podarcis bocagei*. Biomarkers included body condition index, standard metabolic rate, locomotor performance, parasitization, glutathione oxidative pathways and related enzyme activity, lipid peroxidation and liver and testis histology. Few of the various biomarkers investigated provided statistically significant evidence of toxic effect. However, using a weight of evidence approach, we conclude that pesticides are affecting lizards living in the vicinity of pesticide exposed corn agriculture sites. Lizards from these locations present a profile of animals under metabolic stress with reduced condition indices, increased standard metabolic rate, lower incidence of hepatocyte vacuolation, altered iron metabolism, increased activation of GSH oxidation pathways, and even increased prevalence of hemoparasites.

1. Introduction

Lizards have been proposed as ideal sentinels of pollutant induced environmental changes, especially in areas where they are abundant, diverse and have a significant role in ecosystems (reviewed in Lambert (1999, 2005)). Even so, less than 1% of the publications in vertebrate ecotoxicology refer to studies with reptiles and the majority of these focus on turtles or crocodiles (reviewed in Sparling et al. (2010)). Moreover, most studies report only contaminant tissue residue levels and have failed to relate them with the environmental concentrations of the different contaminants and/or the effects of those internal concentrations on individual well being (Hopkins, 2006).

Contaminant effects can be examined at various levels of biological organization, ranging from subcellular expression of proteins up to the viability of populations and ecosystems (Peakall, 1994). Modifications at the population level are usually the crucial focus for ecologists and perceived changes are often the impetus for further studies. Nevertheless, works that examine the effects of contaminants on wild populations are scarce and the lack of data might imply that populations are not being adequately protected (Banks and Stark, 1998). Population effects, where they occur as a direct consequence of exposure to contaminants, generally reflect changes at an individual or subindividual level (Albers et al., 2000; De Coen et al., 2000). Exposure to pollutants has been shown to influence an organism's morphology, physiology, metabolism, and/or DNA integrity, and thus affect its individual ability to acquire resources, grow and reproduce (De Coen et al., 2000; Mitchelmore et al., 2006). Pesticides are one of the main contaminants of concern to the terrestrial environment, and although data on the effects of pesticides in reptiles are scarce, some have detrimental effects on lizard species even at recommended application dosages (e.g. Alexander et al., 2002). Even though a substantial body of knowledge about reptile physiology and ecology exits, few exposure and effect endpoints have been applied to reptile species and frequently they have been adapted from other vertebrate groups (reviewed in Mitchelmore et al. (2006)).

Several authors have proposed integrated approaches with biomarkers at different levels of organization to assess contaminant impacts (Brouwer et al., 1990; Moore and Simpson, 1992; Mann, 2005; Mitchelmore et al., 2006). However, information about the

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impact of pesticides on reptiles is fragmentary and often contradictory. For example, most studies with reptiles have not detected changes in condition indices (CIs) caused by exposure to contaminants (Overmann and Krajicek, 1995; Hopkins et al., 2002). However, McFarland et al. (2008) noticed a weight alteration in several organs of *Sceloporus occidentalis* exposed to trinitrotoluene. CIs have been related with an individual's physiological status, or more specifically its nutritional state; thus it could be expected that a decrease in energetic reserves could influence survival and reproductive success of individuals (van der Oost et al., 2003; Schulte-Hostedde et al., 2005). On the other hand, effects of contaminants on locomotor performance and metabolic energy consumption have been demonstrated experimentally in reptiles (Holem et al., 2006; Hopkins and Winne, 2006; DuRant et al., 2007a,b). Locomotor performance can be considered a culmination of several physiological processes and can play a critical role in an animal's ability to acquire food, avoid predators, successfully mate or defend territories (Bauwens et al., 1995; Holem et al., 2006; Lailvaux and Irschick, 2006). While, the activation of energetically demanding physiological responses (cellular repair mechanisms and increased transport and excretion of contaminants) and metabolic energy consumption can lead to an increase in oxygen consumption (Hopkins et al., 1999).

At a subindividual level, biochemical biomarkers have been used to demonstrate both contaminant exposure and effect in other groups but have rarely been applied in reptiles. This is the case for example, of alterations in antioxidant molecular and enzymatic systems that have been proposed as sensitive exposure biomarkers of cellular oxidative stress in all other vertebrate groups (van der Oost et al., 2003; Abarikwu et al., 2010; Jones et al., 2010; Koivula and Eeva, 2010). Nevertheless, in reptiles the majority of these studies have centered on responses to overwintering and to anoxic conditions (Mitchelmore et al., 2006). Morphological alterations in liver, kidneys and reproductive tissues on the other hand, have been related with pesticide exposure in reptiles (e.g. Guillette et al., 1994; Özelmas and Akay, 1995).

To our knowledge no studies have thoroughly examined the impacts of modern pesticides, and in particular herbicides, on lizards by the use of multiple biomarkers, covering multiple levels of biological organization. The primary hypothesis for this study is that the physiological effects of exposure to a cocktail of pesticides will be manifested within the exposed populations of *Podarcis bocagei*, either as changes in population structure, or as changes in individual biomarkers of exposure and effect. We have adopted a tiered approach that examined: 1. demographics and morphological aspects of natural field populations, the details of which are described in a companion paper (Amaral et al., 2012); and 2. subindividual biomarkers of exposure and toxicity the results of which are presented in this study.

2. Methods

2.1. Animal sampling and maintenance

All lizards used for this study were collected from four agricultural areas in northwestern Portugal in November 2009, before the hibernation period. The animals were collected from different sites to minimize population disturbance. The whole species is highly uniform genetically because of its simple phylogeographic history: with a simple glacial refuge forward by a postglacial northwards expansion (Pinho et al., 2007, 2011). The collection localities included two sites used for corn growing where a set of pesticides was applied (Exposed – Exp 1 and Exp 4) and two organic farming sites (Reference – Ref 1 and Ref 2). The reference sites were a pasture and an organic farming field (several cultures) with no history of chemical application, 20 km east of the exposed sites. Both locations were rural settings with similar geologies, soil types and climates, with no other known sources or histories of contamination. The predominant chemicals detected in the soils of exposed sites were alachlor, bentazone, dicamba, dimethenamid-P, mesotrione and terbuthylazine. Legacy chemicals such as organochlorines or persistent triazines were not detected. A description of the collection sites, including the soil-pesticide profiles is provided in a companion paper (Amaral et al., 2012).

A total of 37 adult male *P. bocagei* (ten from each locality, except for Ref 2 only 7) were caught by noose or hand, transported to the University of Aveiro and housed individually in glass terrariums ($40 \times 20 \times 25$ cm) in a climate control room. Each terrarium contained a terracotta vase ($\varnothing = 16$ cm) that provided a refuge and basking location. Heating was provided by a 25 W incandescent lamp ($8\ h\ d^{-1}$) creating a thermal gradient in the terrarium (25–35 °C). Ambient temperature was maintained at 22 ± 1 °C. Lighting in the climate room was provided by natural sunlight, fluorescent lighting (2×40 W) and a high-pressure sodium lamp (400 W, 7000–12 000 Lx) for $12\ h\ d^{-1}$. Water was provided in shallow dishes and renewed every 2–3 d. Animals were acclimatized for 5 d before the start of the individual experiments. During this period animals were fed mealworms (*Tenebrio molitor* L.). Biomarkers assessed at the individual level included: 1. body condition index; 2. locomotor performance; 3. standard metabolic rate; and 4. predatory behavior (described elsewhere). Following the live animal bioassays (35 d after collection), animals were weighed, killed by cervical dislocation and dissected. Blood, brain, intestine, kidney, liver, testis and thyroid were removed for analysis of the following biomarkers: 1. liver condition index; 2. prevalence and intensity of intestinal and hemoparasites; 3. enzymes involved in the glutathione redox cycle and glutathiones (intestine and liver); 4. lipid peroxidation (intestine and liver); 5. histopathological alterations (liver and kidney); and 6. histological and immunochemical analysis of thyroid–testis axis (described elsewhere).

2.2. Condition indices

When lizards were first brought into the lab, snout–vent length (SVL) and body mass were measured. The general condition of individual lizards was assessed by calculating the Residual Index, based on the residuals from a regression of ln-transformed body mass against SVL (Brown, 1996). An analysis of covariance (ANCOVA) was used to detect differences between individuals from exposed and reference sites, with body mass as dependent variable, type of field (pesticide exposed or reference) as the independent variable and SVL as covariable. After dissection, liver was weighed and mean ln-transformed liver mass, corrected for mean ln-transformed body mass, of individuals from the exposed and reference populations were compared with an ANCOVA. Values of $p < 0.05$ were considered significant in this and the other analyses.

2.3. Locomotor performance

Locomotor performance (sprinting, climbing and clambering) was evaluated by measuring lizard maximum velocity on three different running tracks. Each lizard was fasted for 48 h before the start of the experiment, then weighed, warmed to its optimal temperature (33 °C) (Amaral, unpublished data) and raced three times with at least a 1-h rest interval between trials. Lizards were hand chased through the track and trials were recorded with a video camera (Sony/DCR-HC46 – 25 frames per second), velocities were calculated over each 0.10 m interval. Videos were analyzed with video frame capture software (FrameShots, EOF Productions). Sprint speed was evaluated by measuring the velocity of lizards on a 2×0.1 m sprint track with a cork substrate. Climbing speed

was assessed using a 1 × 0.1 m cork sprint track tilted at a 70° angle. Clambering speed was estimated on 1 × 0.1 m sprint track tilted at a 70° angle covered with a plastic mesh (mesh size = 2 mm) to mimic a vegetation matrix. Following Holem et al. (2008), we calculated mean maximum speed (MMS) as the average of the fastest 0.10 m interval in each of the three replicate sprints, and maximum speed (MS) as the fastest 0.10 m interval for each type of track. In some trials the lizards did not run continuously, these trials were not included in the analysis and the trial was repeated after a 1 h rest period. Quantitative velocity data were examined for statistically significant differences ($\alpha = 0.05$) between exposure groups using MMS or MS as dependent factors in an ANCOVA with type of exposure (exposed, reference) as independent variable, and ln-transformed body mass and ln-transformed SVL as covariates for each type of race.

2.4. Standard metabolic rate

Standard metabolic rate (SMR), the metabolic rate of an inactive lizard, was measured in post-absorptive lizards (fasted for 2–3 d, water provided); a prerequisite condition for SMR measurements (Bennet and Dawson, 1976). The lizards were placed in covered acrylic tubes (230 cm^3) connected to 12 channels of a respirometer (Analytical Developments Co. Ltd., ADC-225-MK3, Hoddesdon, England) housed in a constant temperature room (22 ± 1 °C). An open flow system was employed with a flow rate of 250 mL min^{-1}. Flow rate and CO_2 production were recorded at 30 min intervals during the night, when the animals are inactive and assumed to be in a resting state. Lizards were weighed and randomly assigned to four test groups and a test channel. The lowest 50% of measurements for each individual, corresponding to 18 sequential measurements of CO_2 production representing 9 h during the night, were averaged and used to calculate the molar quantity of oxygen usage by each individual lizard (assuming a respiration quotient of 1:1). A first run with empty channels and one empty channel during each run served to zero the apparatus. To linearize the relationship between body mass and O_2 consumption, data were ln-transformed for all the analyses. Data were plotted as mean ln (O_2 consumption) against ln-transformed body mass (Dorcas et al., 2004). Regression analyses were used to fit lines to exposed and reference data. To examine the effect of pesticide exposition and body mass on SMR, a separate slopes model ANCOVA, with ln-transformed body mass as covariate was used. We used a separate slope model because the groups of lizards have different SMR-mass regression lines.

2.5. Prevalence and intensity of parasites

A blood smear was prepared for each animal, dried at room temperature, fixed in methanol for 1 min and stained with Giemsa (Telford, 2009). Slides were scanned for extraerythrocytic and intraerythrocytic parasites and their prevalences were calculated as the percentage of infected lizards for each type of exposure. Parasite intensity was estimated as the percentage of infected erythrocytes in 2000 cells (Amo et al., 2005). An ANCOVA was performed to test for significant differences ($\alpha = 0.05$) in parasite intensity between lizards from exposed and reference sites, considering SVL as a covariate.

Intestine was rinsed with distilled water under a stereo microscope. The internal contents were fixed in 70% ethanol prior to examination. Helminths were isolated, washed in distilled water, fixed in 96% ethanol and mounted in glycerol according to the techniques described by Jorge et al. (2011). Parasites were identified to family and where possible, genus level.

2.6. Subindividual biomarkers

Frozen samples of liver and intestine were thawed on ice. All tissues were homogenized in 0.1 M Tris–HCl, 0.25 M sucrose and 1 mM EDTA (pH = 7.6) buffer. Homogenates were centrifuged at 10000*g* for 20 min at 4 °C to obtain the post-mitochondrial fraction (supernatant). For the determination of reduced and oxidized glutathione content, a 100-μL aliquot of the supernatant was immediately deproteinized by addition of 100 μL of 10% trichloroacetic acid. Proteins were then removed by centrifugation (10000*g* for 5 min at 4 °C) and the acid supernatant was preserved at −80 °C until determination of reduced and oxidized glutathione. The rest of the supernatant was preserved at −80 °C until enzymatic assays.

Glutathione *S*-transferase (GST, EC 2.5.1.18) was determined spectrophotometrically by the method described by Habig et al. (1974). The reaction medium was 920 μL 100 mM Na-phosphate buffer (pH = 6.5), 20 μL 50 mM 1-chloro-2,4-dinitrobenzene, 50 μL 100 mM reduced glutathione and 10 μL of sample (5 μL of sample in liver). Kinetics was measured for 1 min at 340 nm and tissue activity was expressed as mU mg^{-1} protein using a millimolar extinction coefficient of 9.6 mM^{-1} cm^{-1}. Glutathione reductase (GR, EC 1.6.4.2) activity was measured according to Ramos-Martinez et al. (1983). The reaction medium was 915 μL 100 mM Na-phosphate buffer (pH = 7.0), 25 μL 2.4 mM NADPH, 50 μL 20 mM oxidized glutathione and 10 μL of sample (20 μL for liver). The decrease in absorbance at 340 nm due to NADPH oxidation was measured for 1 min. Tissue GR activity was expressed as mU mg^{-1} protein using a millimolar extinction coefficient of −6.22 mM^{-1} cm^{-1}. Glutathione peroxidase (GPx, EC 1.11.1.9) activity was extremely low in intestine so it was only determined in liver. The glutathione disulfide (oxidized form) formed during the GPx-mediated reaction was converted to its reduced form by the glutathione reductase, and the consumption of NADPH was monitored at 340 nm for 1 min (Lawrence and Burk, 1976). The reaction mixture was composed by 658 μL 100 mM Na-phosphate buffer (pH = 7.5), 40 μL 40 mM reduced glutathione, 32 μL 6 mM NADPH, 20 μL cumene hydroperoxide, 30 μL glutathione reductase and 20 μL of sample. Glutathione peroxidase activity was expressed as mU mg^{-1} protein. All kinetics were carried out at room temperature (20–22 °C) and blanks (reaction mixture free of sample) were periodically read for checking non-enzymatic reaction and enzyme activity was then corrected. Protein concentrations were determined by the Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as a standard.

Reduce and oxidized glutathione levels were fluorimetrically determined according to the method by Hissin and Hilf (1976). To determine the reduced glutathione, acid supernatant was firstly diluted (1:10, dilution factor) with Na-phosphate–EDTA buffer (pH = 8.0). A 100-μL aliquot of the diluted sample was then mixed with 1.8 mL of Na-phosphate–EDTA buffer and 100 μL of 1 mg mL^{-1} ortho-phthalaldehyde (OPA). The derivatization reaction was incubated at 20–22 °C for 15 min and the fluorescence measured at EM = 420 nm and EX = 350 nm. To determine oxidized glutathione concentration, 50 μL of the acid supernatant were treated with 40 μL of 40 mM NEM (N-ethylmaleimide) to prevent oxidation of glutathione. The mixture was incubated at room temperature for 20 min, and then 910 μL 1 N NaOH was added. A 100 μL aliquot was mixed with 1.8 mL of 1 N NaOH and 100 μL of 1 mg mL^{-1} OPA. The reaction mixture was incubated at 20–22 °C for 15 min and fluorescence was read as for reduced glutathione. Standard solutions of reduced (3.27–327 nmol mL^{-1}) and oxidized (1.62–82 nmol mL^{-1}) glutathione were prepared in 0.1 M Na-phosphate–EDTA (pH = 8.0) and handled in the same way as the samples.

Lipid peroxidation (LPO) was estimated by the formation of TBARs according to the method of Ohkawa et al. (1979). An aliquot

of 25 μL of the post-mitochondrial fraction was added to a mixture of 25 μL 8.1% sodium dodecyl sulfate, 187.5 μL of 20% acetic acid and 187.5 μL of 0.8% aqueous solution of TBA (thiobarbituric acid). The mixture was completed with 75 μL of water and incubated at 95 °C for 1 h using a thermoblock. The samples were placed on ice for 10 min and 200 μL of distilled water and 700 μL of a mixture of *n*-butanol:pyridine (15:1 v/v) were added. The tubes were vigorously shaken and centrifuged at 4000 rpm and 4 °C for 10 min. The absorbance of the upper organic layer was read at 532 nm. Lipid peroxidation was expressed as nmol TBARs mg^{-1} protein using a millimolar extinction coefficient of 156 $mM^{-1}cm^{-1}$.

Comparison of the different biomarkers between individuals from reference and exposed locations was done with a *t*-test; when necessary, data were ln-transformed to ensure normality. A Manova based on the Hotelling's *T*-square was used to compare simultaneously all biomarkers per tissue between lizards from exposed and reference areas.

2.7. Histopathological analysis

Liver and kidney were fixed in Davidson's solution for 24 h. Tissues were then washed and stored in 70% ethanol until further processing. Tissues were embedded in paraffin in an automated tissue processor (Microm® STP 120) and 2 μm sections cut on a rotary microtome (Leica® RM 2035). Several staining techniques were employed prior to examination under a light microscope (Olympus® BX51). Tissues were stained with Hematoxylin and Eosin (H&E) for routine examination; with Masson's trichrome to demonstrate fibrosis; with Periodic acid-Schiff (PAS) for the presence of lipids and sugars; and with Perls' Prussian Blue for iron pigments (Bancroft and Stevens, 1996). One representative section per sample was scanned at 400× magnification. This generally involved an examination of more than 20 fields of view. The incidence of histopathological changes was classified through a semi-quantitative scoring system: 0 – normal tissue, 1 – changes in less than 50% of the section, 2 – changes in more than 50% of the section. The proportion of individuals exhibiting the different lesions among exposed and reference sites were compared using Fisher's Exact Tests (Zar, 1996). We used a multiple correspondence analysis (MCA) to graphically present of our data and understand the relationships among the different changes and their incidences according to type of exposure.

2.8. Multivariate analysis

We used discriminant analysis (using the forward stepwise procedure, tolerance = 0.01) to predict group membership. The stepwise procedure uses statistical criteria alone to select the variables that should enter the model to predict to which group (Exposed/Reference) our individuals belong. Only normalized variables were included in the analysis. Variables that were analyzed with co-variables in the univariate analysis were transformed to their residuals. Missing values were replaced with the mean for that variable.

3. Results

3.1. Condition indices

Animals from reference locations had a significantly better health condition than individuals from the exposed sites. Specifically, they had a higher body mass when compared with animals from exposed populations after adjustment for SVL (Fig. 1a, ANCOVA Treatment: $F_{1,34} = 5.62$, $p = 0.023$). No significant difference was observed in liver weight between individuals from exposed and reference sites (data not shown; ANCOVA Treatment: $F_{1,31} = 0.04$, $p = 0.847$).

3.2. Locomotor performance

Normality was not always attained for speed variables. Taking into consideration the skewness and kurtosis estimates we considered that the departure from normality of the ln-transformed variables was not severe and that we could precede with the analysis without resorting to non-parametric statistics.

Sprint speed was slightly reduced in individuals from exposed locations, but both mean maximum sprint speed (Fig. 2, ANCOVA Treatment: $F_{1,33} = 0.24$, $p = 0.629$) and maximum sprint speed (data not shown; ANCOVA Treatment: $F_{1,33} = 0.29$, $p = 0.591$) were not significantly influenced by exposure to herbicides. Likewise, even though there was a slight increase in climbing speed in lizards

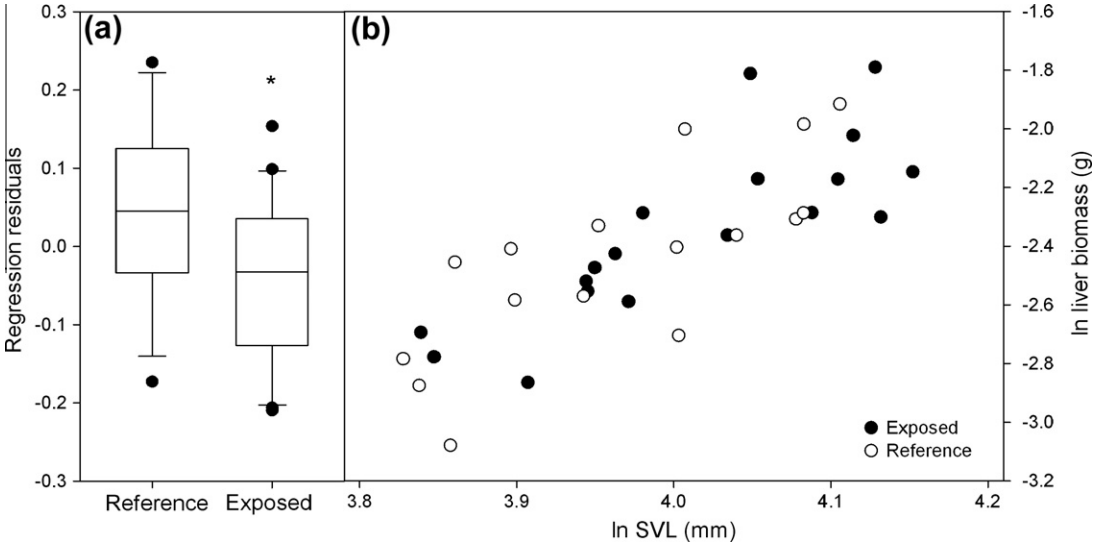

Fig. 1. (a) Body condition of *Podarcis bocagei* from exposed and reference agricultural sites. Body condition index are residual scores from the linear regression of ln-transformed body mass vs. ln-transformed snout–vent length. Box plots show 10th, 25th, median, 75th and 90th percentiles, and outliers ($n = 20$ exposed; $n = 17$ reference). The asterisk indicates a statistically significant difference (ANCOVA; $p < 0.05$). (b) ln-transformed SVL (mm) for *Podarcis bocagei* from exposed and reference agricultural sites plotted against ln-transformed liver biomass (g) ($n = 18$ exposed; $n = 16$ reference).

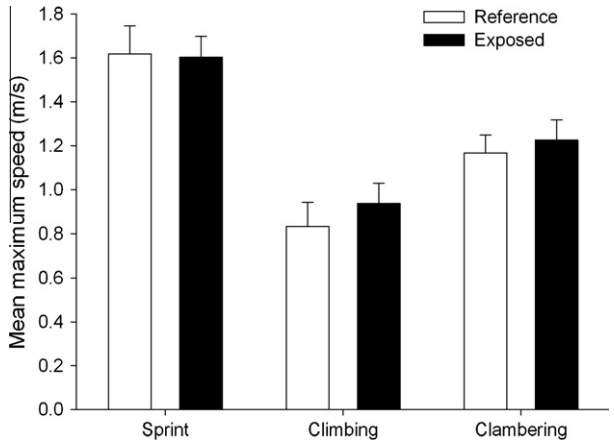

Fig. 2. Mean maximum speed ($m\,s^{-1}$) for *Podarcis bocagei* from exposed and reference agricultural sites in each type of racing track (sprint, climbing and clambering). Bars represent means + standard error; ($n = 20$ exposed; $n = 17$ reference).

from exposed sites, there was no significant variation in mean maximum climbing speed (Fig. 2, ANCOVA Treatment: $F_{1,33} = 0.60$, $p = 0.445$) or maximum climbing speed (figure not shown; ANCOVA Treatment: $F_{1,33} = 0.02$, $p = 0.965$). Clambering speed was also not influenced by exposure to herbicides and no differences were observed for the two variables under study, mean maximum clambering speed (Fig. 2, ANCOVA Treatment: $F_{1,33} = 0.01$, $p = 0.925$) and maximum clambering speed (data not shown; ANCOVA Treatment: $F_{1,33} = 0.04$, $p = 0.844$).

3.3. Standard metabolic rate

Mean SMR among animals collected from the exposed agricultural sites was elevated by 32% (10.1 ± 1.15 μmol h^{-1}, mean ± SE) when compared with animals from reference sites (7.6 ± 0.83 μmol h^{-1}, mean ± SE) (Fig. 3a). An analysis of covariance with body mass as the covariate indicated a marginally insignificant result (Fig. 3a, Separate Slopes Model ANCOVA Treatment: $F_{1,32} = 3.74$, $p = 0.062$). Because lizards were collected from two different exposed sites with different soil pesticide profiles (Amaral et al., 2012), we also examined separately the sub-population of origin. Mean SMR was similar between populations Exp 1 (8.2 ± 1.40 μmol h^{-1}, mean ± SE) and Ref 1 (8.8 ± 0.98 μmol h^{-1}, mean ± SE) but significantly different between animals from Exp 4 (12.0 ± 1.68 μmol h^{-1}, mean ± SE) and Ref 2 (5.7 ± 0.98 μmol h^{-1}, mean ± SE) (Fig. 3b) (ANCOVA Treatment: $F_{3,27} = 3.3$, $p = 0.04$). In the exposed animals the SMR values were independent of size, but in reference populations the differences occurred mainly in small sized animals.

3.4. Blood and intestinal parasites

Prevalence of infection by blood parasites for individuals from exposed areas was 89% and slightly lower for animals from reference areas, 71%. Hemogregarines were the main parasite type but one specimen contained a different parasite, which probably belongs to *Sauroleishmania* Ranque, 1973 (Trypanosomatidae, Trypanosomatida) according to its morphology. The intensity of infection ranged from 1 to 108 parasites in 2000 erythrocytes for individuals from exposed sites (20.5 ± 6.85, mean ± SE) and 1–15 for animals from reference sites (5.3 ± 1.38, mean ± SE). Animals from exposed sites have significantly more parasites than animals from reference sites (Fig. 4, ANCOVA Treatment: $F_{1,25} = 5.2$, $p = 0.03$).

Only one intestinal parasite from the Pharyngodonidae family was found in a lizard from an exposed site.

3.5. Sub individual biomarkers

Table 1 summarizes the mean (±standard deviation) activities of enzymes involved in the glutathione redox cycle as well as glutathione concentrations. For both tissues we did not detect any significant differences in the levels of the glutathione-dependent enzymes between animals from reference and exposed sites (Table 1). For glutathiones there was no difference in intestine. However, we found a significant difference between lizards from the different locations when we compared the reduced glutathione concentrations (Student's t-test, $t = 2.97$, $p = 0.005$) and the ratio of reduced to oxidized glutathione (Student's t-test, $t = 2.79$,

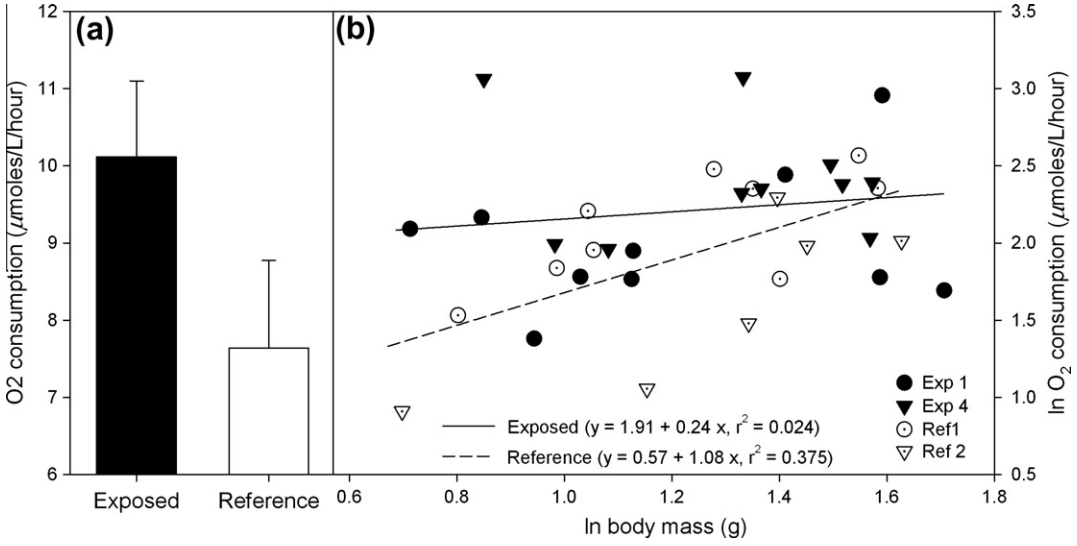

Fig. 3. (a) Mean oxygen consumption rates for *Podarcis bocagei* among lizards sampled from exposed and reference agricultural sites. Bars represent means + standard error; ($n = 20$ exposed; $n = 16$ reference). (b) Mean ln-transformed oxygen consumption rates (μmol h^{-1}) for *Podarcis bocagei* from exposed (Exp 1 and Exp 4) and reference (Ref 1 and Ref 2) agricultural sites plotted against ln-transformed body mass (g). All measures were taken from post-absorptive lizards at 22 °C ($n = 10$ Exp 1; $n = 10$ Exp 4; $n = 9$ Ref 1; $n = 7$ Ref 2).

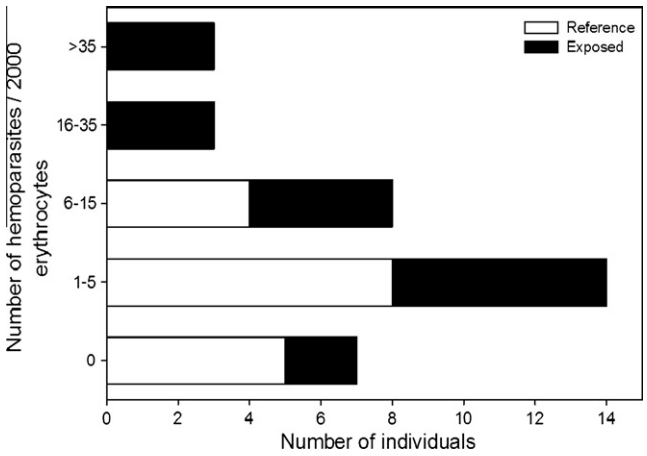

Fig. 4. Intensity of infection by haemoparasites per 2000 erythrocytes for *Podarcis bocagei* ($n = 18$ exposed; $n = 17$ reference).

$p = 0.009$) in the liver. The liver of lizards collected from the exposed agricultural areas had a lower concentration of reduced glutathione than lizards from reference areas. We did not find clear signs of lipid peroxidation in intestine and liver of analyzed individuals (Table 1).

3.6. Liver and kidney histopathology

The histological examinations showed that histopathological changes such as congestion, fibrosis, hepatocyte degeneration and vacuolation and iron pigments were present in the livers of *P. bocagei*, in lizards from both exposed and reference sites. Representative liver sections are shown in Fig. 5. A series of Fisher's Exact Tests (not displayed) did not reveal any difference regarding the occurrence of any of the histological changes observed ($p > 0.05$). Even so, a marginally nonsignificant difference was observed regarding hepatocyte vacuolation, which was more common in lizards from reference sites (Fisher's Exact Test; $p = 0.091$). Our exploratory multiple correspondence analyses showed that hepatocytes from lizards from exposed sites presented intense iron pigmentation with intermediate levels of hepatocyte degeneration but lower levels of hepatocyte vacuolation, compared with lizards from reference sites (Fig. 6).

3.7. Multivariate analysis

The discriminant analysis considered six variables in the model: body condition, number of hemoparasites, standard metabolic rate, liver reduced glutathione, intestinal glutathione reductase and liver glutathione peroxidase (Wilks' Lambda: $F_{6,30} = 4.71$ $p < 0.002$, $N = 37$). Only body condition and number of hemoparasites were statistically different, as in the univariate analyses. The model built with these variables was able to classify correctly 80% of the individuals from exposed sites and 76% of the individuals from the reference sites. Assigning 78% of the individuals to their correct group, based on these six variables (Table 2).

4. Discussion

Mediterranean agricultural areas are treated with pesticides on a regular basis. In Portugal, the last Eurostat report indicated that the use of pesticides has more than doubled since the beginning of the 1990s, with over 12 000 tons of pesticide (active substance) being used in 2003 (Eurostat, 2007). In particular, herbicides can pose a risk to wildlife both because of the volumes used and the areas treated. The impact of herbicides and other pesticides in terrestrial environments remains poorly studied and there is a striking paucity of data on their effects in reptile species that inhabit agricultural fields or adjoining habitats. This study constitutes probably the most comprehensive suite of biomarker responses reported for any reptile. We have used a multi-biomarker approach covering multiple levels of biological organization to look at short term or chronic effects from field exposure to pesticides.

To the best of our knowledge, this is the first study reporting a decrease in body condition index in a reptile inhabiting agricultural areas. No significant differences were found in body condition of tortoises from areas either sprayed or not sprayed with 2,4-D and 2,4,5-T (Willemsen and Hailey, 2001), or in *Gallotia galloti* from agricultural areas compared to those from reference sites (Sanchez-Hernandez et al., 2004). Diminished body condition in lizards has commonly been related with changes in food availability and/or lack of suitable refuges (Ballinger, 1977; Amo et al., 2006, 2007; Pafilis et al., 2009). However, in the present study this is not the case, as we were unable detect any differences in food availability between reference and exposed fields (Simão, 2011). Nor were there any perceived differences in the availability of refuges that may have indicated suboptimal environmental conditions for one or more populations; in all populations, the lizards occupied stone walls of similar construction. No other environmental variable offered an explanation for the observed differences. Temperature, relative humidity, rainfall, and levels of solar radiation are all similar between reference and exposed sites (Amaral et al., 2012), although we cannot discount the presence of other unmeasured chemicals (either natural or anthropogenic) within these respective environments. The loss of body condition can have important consequences for an individual's fitness, including capacity to survive severe winters, ability to compete for breeding opportunities, fecundity, and capacity to fight disease. Indeed, a lower body condition has been related with a compromised immune response and

Table 1
Mean (±standard deviation) activity of glutathione-dependent antioxidant enzymes and glutathione concentrations in intestine and liver of *Podarcis bocagei* from reference and pesticide exposed areas.

Biomarker[a]	Reference		Exposed	
	Intestine	Liver	Intestine	Liver
GST (mU mg^{-1} protein)	75.4 ± 57.29	367.3 ± 115.15	65.7 ± 35.95	319.3 ± 107.70
GR (mU mg^{-1} protein)	36.6 ± 28.92	14.5 ± 5.67	25.7 ± 12.20	12.6 ± 5.31
CHP-GPx (mU mg^{-1} protein)	–	36.9 ± 11.12	–	38.3 ± 7.84
Reduced glutathione (nmol mg^{-1} protein)	10.2 ± 9.23	12.9 ± 5.35	6.4 ± 2.93	8.2 ± 3.74*
Oxidized glutathione (nmol mg^{-1} protein)	11.5 ± 8.63	10.3 ± 4.50	9.3 ± 4.79	11.0 ± 3.83
Total glutathione (nmol mg^{-1} protein)	21.7 ± 16.88	23.3 ± 7.68	15.6 ± 6.98	19.3 ± 5.61
GSH/GSSG	0.9 ± 0.44	1.4 ± 0.58	0.8 ± 0.31	0.9 ± 0.72*
Lipid peroxidation (nmol TBARs mg^{-1} protein)	2.0 ± 1.19	1.5 ± 0.80	1.5 ± 0.72	1.8 ± 1.10

[a] GST – glutathione *S*-transferase, GR – glutathione reductase, CHP-GPx – cumene hydroperoxide-dependent glutathione peroxidase, GSH/GSSG – reduced to oxidized glutathione ratio, TBARs – thiobarbituric acid reactive substances.
* Statistical difference between exposed and reference animals (Student's *t*-test $p < 0.05$).

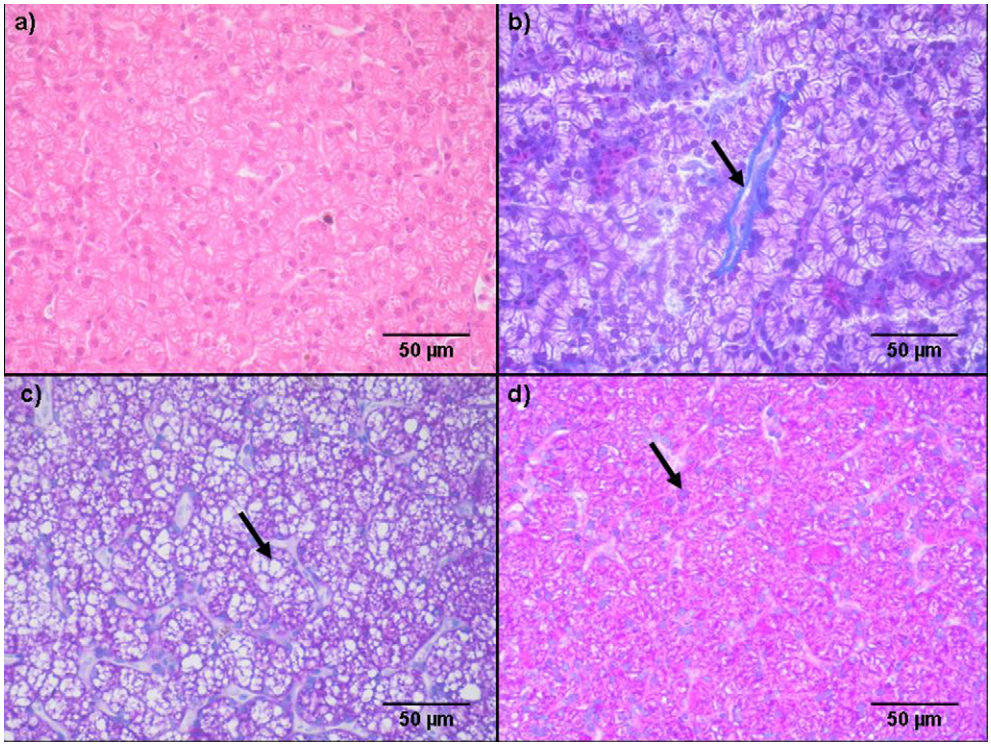

Fig. 5. Representative liver sections of *Podarcis bocagei* with histopathological changes observed, 400×. (a) Liver section of a lizard from a reference site displaying normal appearance, H&E, 400×. (b) Liver section of a lizard from an exposed site displaying fibrosis (arrow), stained with Masson's trichrome. (c) Liver section of a lizard from a reference site displaying hepatocyte vacuolation, (arrow indicates fat accumulation) stained with Periodic acid-Schiff. (d) Liver section of a lizard from a reference site displaying hepatocyte vacuolation (arrow indicates carbohydrates accumulation) stained with Periodic acid-Schiff.

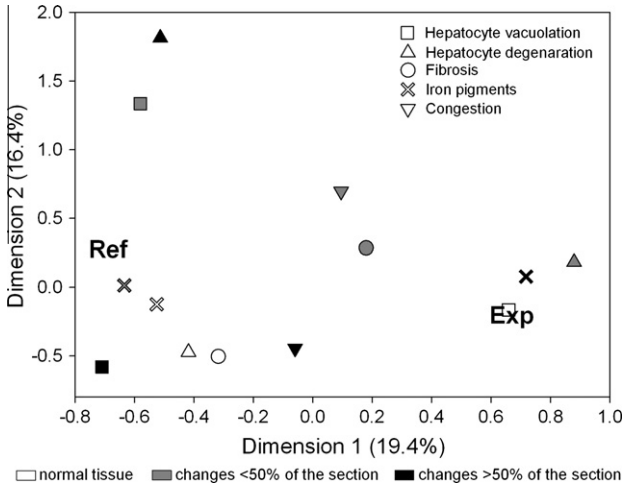

Fig. 6. Results of the multiple correspondence analysis of the incidence of histopathological changes in liver of *Podarcis bocagei* from exposed ($n = 19$) and reference sites ($n = 17$). Each figure represents a histopathological change, and the increasing color the incidence of occurrence of each.

an elevated number of parasites in *Podarcis muralis* (Amo et al., 2006). Depressed immunological responses have also resulted in higher rates of parasite infection in amphibians (Rohr et al., 2008) and reptiles (Olsson et al., 2000; Uller and Olsson, 2003).

P. bocagei (and other continental lacertids) generally display high prevalences of hemoparasites and low rates of intestinal helminth infection (Galdón et al., 2006; Roca et al., 2006; Roca and Galdón, 2010). Despite this, our results still detected an increased intensity of hemoparasites among lizards from exposed sites. Sures (2008) reviewed several aspects of the interaction of parasites and environmental pollutants in the aquatic environment, concluding that parasites have complex and varying implications for host physiology. On one hand, contaminants can affect the immune response of the host and leave it more prone to infection by parasites, while on the other, parasite infection can alter the metabolic response to contaminants (Sures, 2004, 2008). Immunosuppression has also been implicated in several studies examining pesticide exposure in amphibians (review in Mann et al. (2009)) and birds (Mandal et al., 1986). In reptiles no works have ever studied the implications of parasite infection and contaminants.

The liver of an animal provides a window into the history of exposure and response to environmental contaminants (Hinton and Lauren, 1990), and although liver pathologies can rarely be

Table 2
Discriminant function analysis group classification table based on the model that included the variables: body condition, intensity of infection by hemoparasites, SMR, liver GSH, intestinal GR, and liver Gpx.

		Predicted group membership		Total
		Exposed	Reference	
Original group membership	Exposed	16	4	20 (80%)
	Reference	4	13	17 (76.5%)

attributed to exposure to specific contaminants, liver histopathology is a useful biomarker that gives a strong indication of metabolic stress (Stentiford et al., 2003; Richardson et al., 2010). For example, liver fibrosis and congestion have been related with exposure to environmental contaminants in reptiles (Özelmas and Akay, 1995; Rania-Ganser et al., 2003). In the present study, fibrosis and congestion were observed in livers of individuals from both reference and exposed locations, but without any significant differences in prevalence. However, in addition, we detected a tendency for higher levels of hepatocyte degeneration and iron storage in individuals from the exposed populations. Storage of iron in liver has been described in birds and has been associated with altered iron metabolism (Cork et al., 1995), which has been related with starvation or other changes in metabolism (Osborn, 1979).

Other liver indices also point to lower nutritional status among animals from exposed sites. Although not significantly so, lizards from reference sites tended to have slightly larger livers than animals from exposed sites. Liver hypertrophy has been related both with an accumulation of energetic reserves (Kelly, 1993; Santos and Llorente, 2004), and with increased metabolic activity and/or other biosynthetic processes (e.g. Murphy et al., 2006). Our results seem to suggest the occurrence of both conditions. The higher incidence of hepatocyte vacuolation in reference animals was a consequence of lipid and carbohydrate accumulation, confirmed by the PAS staining, which is often described as a result of overfeeding and lack of exercise (Kelly, 1993; Schaffner, 1998). Our animals were collected in mid-autumn at a time when they are gaining energy reserves for winter hibernation, and can be considered a normal physiological condition. While, the lower incidence of hepatocyte vacuolation observed in exposed animals, although not statistically significant, indicates that these animals were not accumulating energetic reserves (carbohydrates and lipids). This might be a sign of malnutrition as a consequence of reduced capacities to obtain food and/or increased metabolic demands for detoxification.

As indicated earlier, a shortage of food resources or other physical environmental stresses did not account for the depleted nutritional status of lizards from exposed sites, which must otherwise be explained by increased energetic demands. Indeed, exposed lizards appeared to have elevated oxygen consumption; 32% higher than animals from reference locations. Furthermore, when the four groups of lizards (i.e. two groups from reference sites (Ref 1 and Ref 2) and two groups from exposed sites (Exp 1 and Exp 4) – for site descriptions see Amaral et al. (2012) were examined individually, there was a statistically significant elevation in oxygen consumption among lizards from Exp 4 (an increase of more than 100% compared to Ref 2 lizards), and it was this site that had the highest total soil concentration of herbicides in Spring 2009. It has been estimated for ectotherms that more than 80% of the total energy budget is required for maintenance and essential processes (Congdon et al., 1982; Brown et al., 1992). Therefore, an increase in the standard metabolic rates for maintenance requirements has to be compensated either by an increase in energy assimilation or by a decrease in energy available for production (Hopkins et al., 1999). We can hypothesize that the energy balance in animals from exposed sites will occur through a reduction of energy available for growth, storage or reproduction, which can ultimately affect population dynamics. In summary, liver condition and histology and the increased standard metabolic rate are congruent with a decreased body condition in exposed individuals.

Several studies with reptiles have suggested that changes in locomotor performance can be used as an indicator of exposure to AChE-inhibiting pesticides despite recognizing that it probably it is not a very sensitive parameter (DuRant et al., 2007b; Holem et al., 2008). In the present study, we also observed no differences in locomotor capacities between lizards from exposed sites and those from reference sites. However, in our experiments, the lizards were not (as far as we are aware) exposed to AChE inhibitors (e.g., carbamate or organophosphorus insecticides), and there were not likely to be any direct effects on cholinergic neural pathways, making comparison with the aforementioned studies tenuous. However, as was the case with previous studies (DuRant et al., 2007b; Holem et al., 2008), it is important to recognize that in the locomotor tests, the chasing of lizards triggered a flight response, and it seems likely that the "fight or flight" responses are less affected by an animal's general fitness, at least over short distances. Therefore, a better indicator of physiological impairment may be a challenge test that involves multiple neuromuscular systems, other than simply a sympathetically controlled reflexive flight response. The same animals used in these locomotor tests were also used in tests that examined their capacities to forage for prey. The results of those tests will be presented elsewhere.

The endogenous thiol, glutathione (GSH), and the enzymatic systems that contribute to its cellular redox status (e.g., GST, GR or GPx activities) are often determined in pollutant exposed organisms as a measure of their oxidative status in the presence of pro-oxidant exposure (van der Oost et al., 2003; Koivula and Eeva, 2010). Glutathione is an important scavenger for electrophilic compounds that conjugates with GST in contaminant metabolism. A decrease in reduced glutathione (GSH) is usually accompanied by an elevation of its oxidized disulfide form (GSSH) and a concomitant decrease of the GSH/GSSG. These changes in the glutathione redox balance are considered a sign of oxidative stress (Storey, 1996). We compared the response of selected biomarkers of oxidative stress among the lizards caught from the reference and exposed areas. Lizards from the exposed agricultural areas did indeed display a significant reduction in GSH and GSH/GSSG ratio in the liver but no differences were observed in the activity of enzymes involved in its redox cycle such as GR or GPx activity. Short-term exposure to pesticides that cause oxidative stress such as the herbicide alachlor could explain this rapid change in the GSH/GSSG ratio. Indeed, alachlor is the only agrochemical detected in our study area for which documented effects on liver biomarkers had been previously reported for other vertebrate species. For example, this herbicide caused an induction of GST activity in crucian carps, although such an enzymatic induction was not dose dependent (Yi et al., 2007). An increase of GSH contents was also observed in the common carp and *in vitro* in rat and human cell lines (Mikula et al., 2009). All animals used for this study were sampled in November 2009, approximately 6 months following herbicide applications in the previous spring. Notwithstanding the continued exposure to the more persistent chemicals such as alachlor, we expect that most of the chemicals had degraded substantially by the time the animals were collected. The animals were also maintained for 35 d in a clean laboratory environment prior to tissue collection. The activities of these enzymes are in a constant state of flux and their reported activities do not necessarily represent the transient responses that will occur following exposure. However, the observed changes in GSH/GSSH ratio suggest that a longitudinal study before and after exposure may be warranted.

For reptiles, oxidative stress responses have mostly been studied in relation to natural stressors related with hibernation and freezing tolerance. Storey (1996) considered that species that do not experience prolonged periods of anoxia respond to oxidative stress by increasing the antioxidant enzyme levels to respond to the anticipated increase in reactive oxygen species. Under anoxic conditions, GSH levels increased in garter snakes while GR and GST levels were mostly unaffected and the activities of several antioxidant enzymes in different tissues of red-eared slider turtles decreased (reviewed in Hermes-Lima and Zenteno-Savín (2002)). Comparing the different tissues, lizard liver presented higher levels of GST, which is in accordance with other studies in reptiles

(Voituron et al., 2006; Valdivia et al., 2007), followed by intestine. Both tissues (liver and intestine) displayed similar concentrations of glutathiones. Similar levels of lipid peroxidation were found between tissues without any effect of exposure, but taking into consideration that lipid peroxidation has been shown to increase with advancing age in a similar short-lived species (Jena et al., 1995), these data should be interpreted with caution. Despite the decrease of GSH/GSSG ratio in the liver of lizards from exposed agricultural areas, it is noteworthy that statistical differences in the variations of this index of oxidative stress could not be detected in the intestine. Cellular concentrations of GSH are generally higher than GSSG in normal physiological conditions. However, we found that the levels of both glutathiones were similar in the intestine despite the care taken to avoid any oxidation of glutathione during sample handling and during the homogenization procedure.

5. Conclusion

Field situations are ecologically complex but our results suggest a difference between animals from reference and exposed locations. The absence of strong statistically significant indications of toxicity highlights the need for far greater replication, which is inherently difficult in field studies of this nature. Greater sample size would allow the detection of true physiological differences and overcome inherent variability that occurs as a consequence of ecological or genetic differences. These results also reinforce the need to use an integrated series of different biomarkers of exposure and effect. In the present study, it is clear that some biomarkers have indicated potential toxicity among lizards exposed to a cocktail of different pesticides, even though no individual biomarker has provided categorical evidence for toxic effect, and indeed the cumulative value of several marginally significant or marginally insignificant results needs to be weighed up cautiously due to the high number of tests performed. However, using a weight of evidence approach, we conclude that lizards living in the vicinity of corn agriculture are being affected by exposure to pesticides in their environment. Furthermore, the suite of biomarkers that did indicate toxicity – reduced condition indices, increased SMR, lower incidence of hepatocyte vacuolation, increased activation of liver GSH oxidation pathways, and even increased prevalence of hemoparasites – provide a coherent profile of animals under metabolic stress. The discriminant analysis further reinforced these results by correctly classifying more than 75% of the animals using body condition, SMR and liver GSH oxidation pathways responses.

Overall, those lizards living in locations exposed to various herbicides are less ecologically fit than those living in reference sites. This exposure, and the toxicity associated with it do not appear to be affecting populations (see Amaral et al., 2012). However, if the added stress of pesticide toxicity takes animals closer to their survival thresholds, then they are likely to be more vulnerable to stochastic events (e.g. unusually cold weather, draught, disease, etc.). In summary, our study facilitated the validation of different biomarkers at several levels of biological organization for field exposed lacertid lizards and reinforced the need of a multi-biomarker approach in these early days of reptile ecotoxicology. *P. bocagei* was confirmed to be a suitable bioindicator species and the results obtained stressed the need to include reptiles in environmental risk assessments, as they might be vulnerable to chemical contamination in combination with other natural stressors.

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