

The usefulness of mesocosms for ecotoxicity testing with lacertid lizards

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Abstract. Mesocosms (i.e., outdoor, man-made representations of natural ecosystems) have seldom been used to study the impact of contaminants on terrestrial ecosystems. However, mesocosms can be a useful tool to provide a link between field and laboratory studies. We exposed juvenile lacertid lizards for a period of over one year to pesticides (herbicides and insecticides) in mesocosm enclosures with the intention of validating field observations obtained in a previous study that examined the effects of corn pesticides in *Podarcis bocagei*. Our treatments replicated field conditions and consisted of a control, an herbicides only treatment (alachlor, terbuthylazine, mesotrione and glyphosate) and an herbicides and insecticide treatment (including chlorpyrifos). We used a multi-biomarker approach that examined parameters at an individual and sub-individual level, including growth, locomotor performance, standard metabolic rate, biomarkers of oxidative stress, esterases and liver histopathologies. Although mortality over the course of the exposures was high (over 60%), surviving individuals prospered relatively well in the mesocosms and displayed a broad range of natural behaviours. The low numbers of replicate animals compromised many of the statistical comparisons, but in general, surviving lizards exposed to pesticides in mesocosm enclosures for over one year, thrived, and displayed few effects of pesticide exposure. Despite the difficulties, this work acts as an important stepping-stone for future ecotoxicology studies using lizards.

Keywords. Pesticides, reptiles, biomarkers, terrestrial ecotoxicology, alachlor, chlorpyrifos.

INTRODUCTION

Laboratory toxicity testing using single species is by far the most commonly employed approach for establishing the toxicity of chemicals to both aquatic and terrestrial organisms. Laboratory studies are relatively inexpensive and allow a high degree of control over various exposure parameters and interactions, like dose, test duration or temperature. However, their simplistic approach brings several limitations (reviewed by Banks and Stark, 1998). For example, terrestrial organisms such as lizards are not continuously in contact with the exposure media, and simulation of toxicant exposure in the laboratory that would simulate real-world conditions is a difficult task. At the other end of the broad spectrum of studies encompassed by the discipline of ecotoxicology, field studies are crucial to understand site-specific questions. However, field studies are difficult to replicate and interpret because of the complexity and variability of natural habitats, involving multiple, frequently synergistic, causative factors (Van den Brink et al., 2005). Furthermore, the effects of a contaminant in natural situations can be altered or masked by the presence of biotic (competition, predation) or abiotic (temperature, humidity, rainfall) factors, or by interaction with other contaminants (reviewed by Banks and Stark, 1998). In this context, mesocosms can provide a bridge between field studies and overly contrived laboratory manipulations, bringing both environmental realism and control, and serving as a validation tool for both types of studies.

Mesocosms have been defined as outside enclosures with a semi-controlled ecosystem that provide an environment that can be replicated, with some degree of realism (Odum, 1984; Van den Brink et al., 2005). Organisms are contained to ensure re-sampling but are subject to natural conditions, species interactions, etc. Most importantly, contaminated and uncontaminated environments can exist in the same place, and experience exactly the same climatic and geochemical conditions. Mesocosm enclosures have been used regularly in aquatic environments, for example to study the responses of amphibians to contaminants (Rowe and Dunson, 1994). In the terrestrial environment however, they have been used to a far lesser extent (Boone and James, 2005). Still, they can be useful to examine the effects of pesticides on terrestrial vertebrates because the entire terrestrial environment can be contaminated, thereby replicating a realistic exposure scenario.

In the case of reptiles, mesocosms have rarely been employed to study the impact of contaminants (e.g., Alexander et al., 2002; Guirlet and Das, 2012). However, European lacertid lizards have been proposed as potential model species for reptile ecotoxicology (e.g., Mann et al., 2006; Marsili et al., 2009) and are ideal candidates for mesocosm studies. Two characteristics of lacertid lizards that make them well suited for mesocosm studies are their small size and small home-range.

Recently, we completed a field study that examined various population indices and biomarkers of pesticide exposure and effect among several sub-populations of *Podarcis bocagei* living in field margins of corn pastures where several pesticides were annually applied, or within agricultural fields characterized by an absence of pesticide use (Amaral et al., 2012a, b). These studies provided evidence for an adverse effect of pesticide exposure at the individual level, although lizard populations seemed to persist in the exposed fields despite evidence for toxic effects. The goal of the present study was to expose *P. bocagei* lizards to the same cocktail of pesticides found in our previous field surveys and thereby reinforce the findings of those studies.

MATERIALS AND METHODS

Animals

Podarcis bocagei is a small insectivorous lacertid endemic to the north-western Iberian Peninsula that occupies a variety of habitats, from wide open forests with brushwood to coastal dunes and agricultural areas (Galán, 1986). Hatchlings were reared from eggs laid in the lab by females collected in coastal dune system of Mindelo (Vila do Conde) on the north-western coast of Portugal during April-May 2009. Gravid females were maintained in pairs in terraria (40 x 20 x 25 cm), in a climate room (22 ± 1 °C) with food (mealworms, *Tenebrio molitor* larvae) and water provided *ad libitum*. Snout-vent length of each female was measured when they were brought into the lab. Each terrarium contained a terracotta vase (diameter = 16 cm) that provided refuge and a basking location, and a 25 W incandescent lamp that created a thermal gradient in the terrarium (25–35 °C; 8 h day⁻¹). Lighting was provided by natural sunlight, fluorescent lighting (2 x 40 W) and a high pressure sodium lamp (400 W, 7000-12 000 Lx) for 12 h day⁻¹. Every night females were kept in individual boxes filled partially with clean commercial soil where they could lay eggs. The boxes containing newly laid eggs were placed in an incubator at 33 °C until hatching. After hatching, juvenile lizards from the same clutch were kept together in similar conditions to those of females until the last hatchling attained one month of age. Hatchlings were fed mealworms, isopods (*Porcellionides pruinosus*) and fruit flies (*Drosophila melanogaster*). Hatchlings used for the experiment had an average age of 65.5 days (32-88 days, minimum and maximum) and size of 27.6 mm (snout-vent length, 23.7-32.8 mm, minimum and maximum).

Study design

Our experiment was conducted from September 2009 to November 2010 in the grounds of the University of Aveiro (80 km south of the collection point), encompassing the first year of development of the hatchlings, including a hibernation period. In early 2009, we constructed 12 enclosures (100 x 150 x 100 cm), with four replicates per treatment: Control (Ctr), Herbicides (Herb), Herbicides + Insecticide (Herb+Ins). The mesocosms were constructed from transparent acrylic (4 mm) joined at each corner with stainless steel brackets, and buried 50 cm in soil to prevent lizard escape by burrowing (Fig. 1A). The bottom of each enclosure was filled with a layer of sand and soil from the same location to ensure adequate drainage. This was then capped with an upper layer of commercial soil (Siro® universal substrate). In the spring of 2009, turnip (*Brassica rapa*) was planted in all mesocosms and spontaneous vegetation was allowed to emerge (Fig. 1B). Each mesocosm was furnished with three terracotta building bricks with multiple cavities to provide refuge; five plastic tubes dug into the soil (up to 0.15 m) to simulate burrows and a water and food dish. The buried tubes provided an insulated refuge to facilitate survival over winter. A net (mesh size 10 mm) covered each mesocosm to prevent the entrance of predators (e.g., birds, cats).

We used the same pesticides as those routinely applied in the corn-growing regions of north-western Portugal (Amaral et al., 2012b) and they were applied at the same application rates as recommended for corn agriculture. In the Herb treatment, we applied four herbicides with a 2 L hand-held sprayer: Controller-T® (336 g L⁻¹ alachlor and 144 g L⁻¹ terbuthylazine - SAPEC, 7 L ha⁻¹), Calisto® (100 g L⁻¹ mesotrione - Syngenta, 1.5 L ha⁻¹) and Roundup® (360 g L⁻¹ glyphosate - Monsanto, 4 L ha⁻¹). In the Herb+Ins treatment, we applied Ciclon 5G® in granular formulation (5% p/p chlorpyrifos - SAPEC, 60kg ha⁻¹) in addition to the herbicides. Pesticides were applied during June of 2009 and April 2010 in all mesocosms (except Ctr enclosures).

Three hatchlings, individually marked by toe clipping, were randomly assigned to each mesocosm and treatment group and introduced to the enclosures in September 2009 (day 0), approximately three months after the initial pesticide application in June 2009. Animals were recovered from

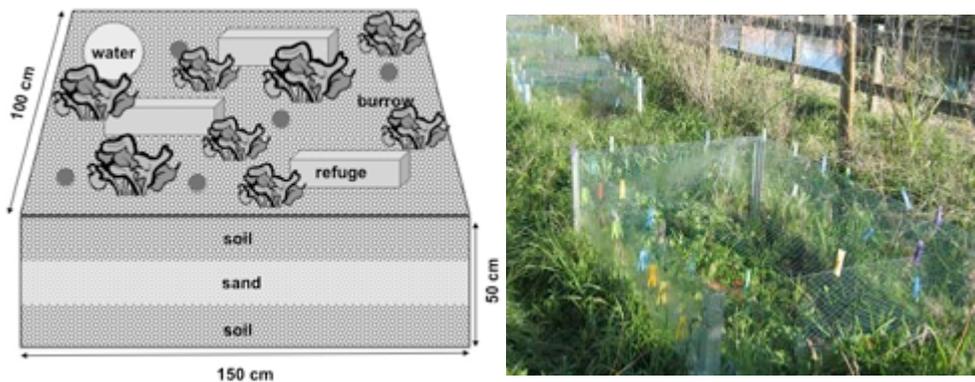


Fig. 1. A) Scheme of the mesocosm. B) Photo of one of the enclosures in Spring 2010, before pesticide application.

the mesocosms at two interim time-points. The first was in March 2010 (day 190), one month before a second pesticide application, and the second in April 2010 (day 220) shortly after pesticide application. The experiment was concluded in October 2010 (day 413) (Fig. 2). Lizards could eat wild prey or mealworms provided twice a week. Provided mealworms were placed during four days in a portion of soil collected from control or treated enclosures in day 0 or after pesticide application in 2010. At each of the two interim time points, lizards were assessed for growth and locomotor performance. At the end of the experiment, lizards were measured, rendered slightly torpid by cooling, sacrificed by decapitation, and dissected. Blood was immediately collected with a micropipette from the exposed trunk, centrifuged for 10 min at 800 rpm and 4 °C to obtain plasma, which was frozen in liquid nitrogen. Intestine, two thirds of the liver and brain were similarly frozen in liquid nitrogen and stored at -80 °C prior to biochemical assays. The remaining third of the liver was fixed in Davidson's solution for 24 h, washed in distilled water and stored in 70% ethanol until histopathological analysis. All procedures in this study complied with Portuguese animal ethics guidelines as stipulated by *Direcção Geral de Veterinária* and *Instituto da Conservação da Natureza e Biodiversidade*.

Pesticide concentrations in soil

Soil samples were analyzed for the applied pesticides on four occasions: at the beginning of the experiment, on days 190 and 220 and at the end. At the beginning of the experiment we collected one sample per enclosure. In the subsequent samplings we decided to collect three replicate soil samples per enclosure to account for variability within enclosures. The location of the sampling points in each enclosure was randomly selected from a 9 square grid. Samples were preserved in clean, new, 200 ml glass vials, covered with Parafilm® and sent to Marchwood Scientific Services (Southampton, UK) for QuEChERS chemical analysis (Anastassiades et al., 2003).

Growth and body size

Snout-vent length (SVL) and body mass were measured (to the nearest 0.01 mm and 0.1 g, respectively) on four occasions: day 0, 190, 220 and 413. Mean growth rates (SVL; cm day⁻¹) between each period were calculated for each treatment.

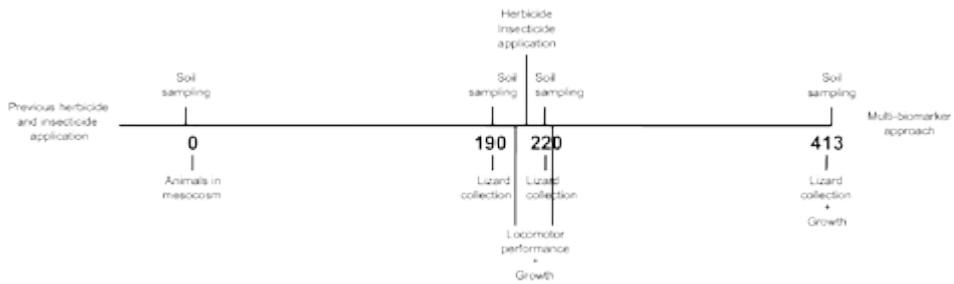


Fig. 2. Timeline (days) of study.

Locomotor performance

Locomotor performance was evaluated twice, before and after pesticide application in 2010 (day 190 and 220) by measuring lizard maximum sprint speed on a cork substrate (2×0.1 m sprint track). Each lizard was fasted for 48 h before the start of the experiment, weighed, warmed to its optimal temperature of 33 °C (Amaral, unpublished data) and raced three times with at least one hour rest interval between trials. Lizards were hand-chased through the track and trials were recorded with a video camera (Sony/DCR-HC46 – 25 fps). Following Holem et al. (2008), mean maximum speed (MMS) was calculated 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 in any of the trials. Trials in which lizards did not run continuously were repeated.

Standard metabolic rate

Standard metabolic rate (SMR), the metabolic rate of an inactive lizard, was measured at the end of the experiment (day 413). Lizards were fasted for 2 days (water provided) before the measurements, as a post-absorptive metabolism is a prerequisite condition for SMR measurements (Bennet and Dawson, 1976). 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 one of two test groups (it was not possible to run all animals at the same time) 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 used 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 calibrate the apparatus.

Biomarkers of oxidative stress

Antioxidant enzymes were determined in brain, intestine and liver. Glutathione *S*-transferase (GST, EC 2.5.1.18) activity was determined spectrophotometrically according to Habig et al. (1974). Specific activity was expressed as mU/mg protein using a millimolar extinction coefficient of 9.6

$\text{mM}^{-1} \text{cm}^{-1}$. Glutathione reductase (GR, EC 1.6.4.2) activity was measured following the method described in Ramos-Martinez et al. (1983). The decrease in absorbance at 340 nm due to NADPH oxidation by reduced glutathione was measured for 1 min, and a millimolar extinction coefficient of $-6.22 \text{ mM}^{-1} \text{cm}^{-1}$ was used for the specific activity calculations. All kinetics were carried out at room temperature (20–22 °C) and blanks (reaction mixture free of sample) were periodically checked for 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.

Concentrations of reduced glutathione and oxidized glutathione were fluorimetrically determined according to the method of Hissin and Hilf (1976). Reduced glutathione was determined by incubation of the deproteinized samples in the presence of 1 mg ml^{-1} *ortho*-phthalaldehyde (OPA) in Na-phosphate (0.1 M)- ethylenediaminetetraacetic acid (EDTA, 5 mM) buffer (pH 8.0). Determination of the oxidized glutathione concentrations required a preliminary incubation step with N-ethylmaleimide to prevent glutathione oxidation, and 1 N NaOH was used instead of the phosphate-EDTA buffer. In both methods, the reaction mixture was incubated for 15 min at 20–22 °C and the fluorescence was read at excitation wavelength (EX) = 350 nm and emission wavelength (EM) = 420 nm. Quantification was performed using a set of external standards of reduced (3.27 to 327 nmol ml^{-1}) and oxidized (1.62 to 82 nmol ml^{-1}) glutathione, which were prepared in the same manner as the samples.

Lipid peroxidation was included as a measure of oxidative damage. The method described in Ohkawa (1979) was used to estimate lipid peroxidation through the formation of thiobarbituric acid reactive substances (TBARs). Lipid peroxidation was expressed as $\text{nmol TBARs mg protein}^{-1}$ using a millimolar extinction coefficient of $156 \text{ mM}^{-1} \text{cm}^{-1}$.

Esterases

Carboxylesterase (CbE) activity was measured in the intestine, liver and plasma using two substrates: α -naphthyl acetate (α -NA) and the 4-nitrophenyl valerate (4-NPV). Hydrolysis of α -NA was performed following the method by Gomori and Chessick (1953) as adapted by Bunyan et al. (1968), by which the formation of α -naphthol occurs in a reaction medium that contains 25 mM Tris-HCl (pH 7.6), 2 mM α -NA and the sample. The hydrolytic reaction was stopped after 10 min by addition of 2.5% (w/v) SDS and subsequently 0.1% Fast Red ITR in 2.5% Triton X-100. The solutions were allowed to stand for 30 min in the dark and the absorbance of the naphthol–Fast Red ITR complex was read at 530 nm ($\epsilon=33.225 \times 10^3 \text{ M}^{-1} \text{cm}^{-1}$). Hydrolysis of 4-NPV by CbE activity was measured according the method by Carr and Chambers (1991). The reaction mixture contained 1 mM 4-NPV, 50 mM Tris-HCl (pH 7.5) and the sample. After 15 min, the reaction was stopped by adding a solution of 2% (w/v) SDS and 2% (w/v) Tris base. The 4-nitrophenolate liberated was read at 405 nm and quantified by a calibration curve (5–100 μM). Cholinesterase activity was determined using the substrates acetylthiocholine iodide (AcSCh) and *S*-butyrylthiocholine iodide (BuSCh) as described by Ellman et al (1961). Specific ChE activity was expressed as mU mg^{-1} of protein using a molar extinction coefficient of $14.15 \times 10^3 \text{ M}^{-1} \text{cm}^{-1}$ (Eyer et al., 2003).

Liver histopathology

Samples were embedded in paraffin and 2 μm sections cut on a rotary microtome (Leica RM 2035). Tissues were stained with Haematoxylin and Eosin (H&E) and examined under a light microscope (Olympus BX51) equipped with an Olympus camera. Liver sections were also stained with Masson's trichrome to assess liver fibrosis; with Periodic acid-Schiff (PAS) for the presence of lipid or sugar accumulation, and with Perls' Prussian Blue to verify iron pigmentation (Bancroft and Stevens, 2002). One representative section per sample was scanned at 400x magnification. This gen-

erally involved an examination of a minimum of 25 fields of view. The incidence of histopathological changes was classified according to 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.

Statistical analyses

All randomizations were performed using the function rand of Excel. The results obtained from the treatment groups were compared to the control using analysis of variance (ANOVA), repeated measures analysis of variance or/and analysis of co-variance (ANCOVA) followed by Dunnett's or Duncan's multiple comparison post-hoc tests. Treatment was the dependent variable, and SVL or body mass were used when appropriate as covariates to check for significant interactions. Differences in the incidence of histological changes between treatments were compared with the control using Fisher's exact tests (Zar, 1996). Differences in SVL were compared using Student's t-test or the non-parametric Mann-Whitney test when data were not normal. Statistical analyses were performed using STATISTICA 7 (Hill and Lewicki, 2006), for Windows. Values of $P < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Lizard survivorship

Lizard survivorship was poor in all treatment groups. Of the 36 original lizards, 30% did not survive the winter and less than 40% survived during the entire period, with similar levels of mortality occurring among all groups and mesocosms. Mortality was not related to size of the lizards, both in the first interval (Mann-Whitney $U = 312.5$, $P = 0.99$, $n = 36$) and in the last interval (Student's $t = -0.7$, $P = 0.46$, $n = 23$). In the second interval, only two lizards did not survive. These were two of the smallest animals but an accurate statistical comparison is not possible because of the small sample size. High mortality rates after hibernation for oviparous species were described for several lizards (Pike et al., 2008), including lacertids – 92% of males and 86% of females (Civantos and Forsman, 2000), and in particular among *P. bocagei* juveniles – 52% (Galán, 1999, 2004). Despite the high mortality rates, the remaining lizards prospered relatively well in the mesocosms, and displayed a broad range of natural behaviours. At the end of the exposure period, we recovered the following individuals from each treatment: Ctr – 3; Herb – 4; Herb+Ins – 6. The small number of individuals per treatment prevented us from using the enclosures as replicates and all the statistical analyses are based on individual responses. We were also unable to test sex-related variations in our dependent variables. Nevertheless, as we were dealing with juveniles and sub-adults we can expect that sex differences will not be overly relevant.

Levels of pesticides in mesocosm soil

Active ingredients of the applied pesticides were found in varying concentrations during the experimental period (Fig. 3). Pesticide levels in control soil were always below

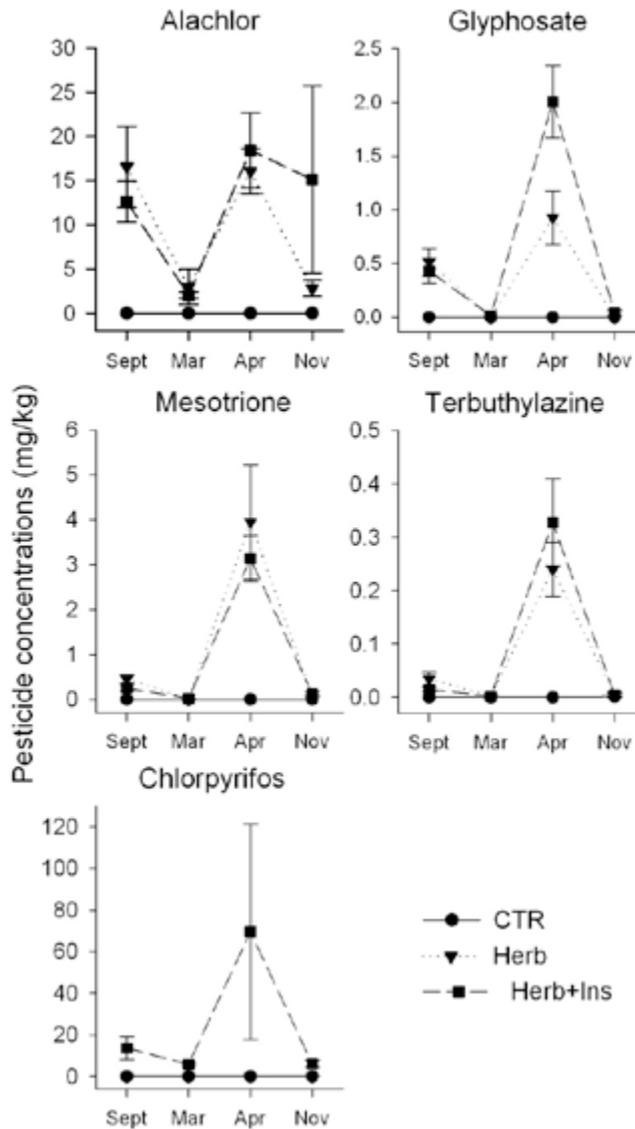


Fig. 3. Average pesticide content of soils ($\mu\text{g kg soil}^{-1}$) in the three treatment groups, Ctr, Herb and Herb+Ins. Data points represent the mean of the mean values for each enclosure of the three replicate sampling points. Error bars represent standard error). Limit of Detection - $0.5 \mu\text{g kg}^{-1}$.

quantification limits. As the study involved manual spraying of small volumes of herbicides, some discrepancy between and within mesocosms was expected. At day 0, more than three months after the initial application of pesticides, there were residues of all applied

herbicides in the Herb and Herb+Ins mesocosms, with alachlor occurring at the highest concentration. In the Herb+Ins treatment there was also a high concentration of chlorpyrifos. At day 190, 10 months after the first pesticide application, all the concentrations had decreased and only alachlor and chlorpyrifos were detectable (in Herb and Herb+Ins, respectively). Half-lives for these products in soil are of 5-42 days for alachlor (Mackay et al., 2006) and 30-60 days for chlorpyrifos incorporated in soil (Racke et al., 1996). After the re-application at day 220, pesticide concentrations reached their maximum for the experiment, and again decreased by the end of the study (day 413), with the exception of alachlor in the Herb+Ins treated mesocosms. The high mean concentration for alachlor was influenced by a single replicate soil sample in one of the enclosures. These high variations between or within enclosures may be the result of slightly different microclimatic conditions inside each mesocosm (e.g., shading). The half-life of a chemical has been shown to depend on a number of factors, including climate and soil characteristics (Gevao et al., 2000). In particular, soil microorganisms (bacteria and fungi) primarily degraded alachlor through conjugation with Glutathione S-transferases (GST) (reviewed by Sette et al., 2004). Soil microorganism activity is dependent on soil temperatures; thus, it can be expected that if that area of the enclosure was shaded for longer periods, soil temperature was likely to be lower and the rate of alachlor degradation would be lower when compared with the other sampling points in the same enclosure or other mesocosms.

We can expect that lizards in our mesocosm will be exposed to these products by dermal exposure and ingestion of contaminated food or soil. The turnover of insects and other invertebrates in the enclosures is expected to be high, which could decrease the rate of exposure through trophic transfer. However, the inclusion of pre-contaminated mealworms within the experimental design ensured that the lizards were exposed to some contaminated prey items.

Variation in life-traits

Galán (1997) demonstrated for *P. bocagei* that the size of the mother influences the number of eggs and hatchlings but not their size at hatching. Our results also found no correlation between the size of the mother and the size of individual hatchlings at the beginning of the experiment. This, together with the random distribution of individuals in treatment groups guaranteed that variations in body size could not be attributed to variations at hatching, and more specifically, to the size of the mothers.

Lizard body size increased in all treatments, achieving sizes equivalent to those found in nature (Galán, 1997; Carretero et al., 2006). At the end of the experiment, all surviving individuals had attained adult size (Fig. 4A). Growth, expressed as change in body size, is an integrative process that can be related with several population level parameters and influence population structure and dynamics (Gaston et al., 2001), and growth rate can be interpreted as an index of individual fitness. Failure to grow and mature within a specified time would indicate that animals were unable to obtain and assimilate the resources necessary for maintenance as well as allocate energy for growth, storage of energetic reserves or reproduction (Brown et al., 1992; Civantos and Forsman, 2000; Mitchelmore et al., 2006). This would be especially critical before hibernation, as it is likely the main determinant of juvenile survival (Civantos and Forsman, 2000).

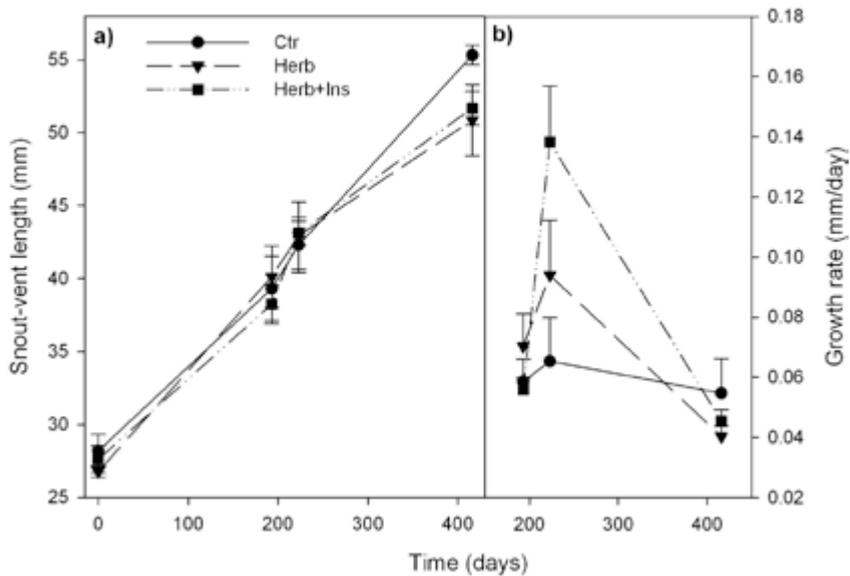


Fig. 4. A. Mean snout-vent length (mm) of *Podarcis bocagei* lizards raised in Ctr, Herb and Herb+Ins mesocosms. Measures were taken when lizards were first introduced in the mesocosm (Day 0), and at three subsequent sampling dates (Day 190, 220, 433). Mean \pm standard error. The n value varied over time because of lizard mortality. B. Mean growth rate (mm day⁻¹) of *P. bocagei* lizards raised in Ctr, Herb and Herb+Ins mesocosms over the three time intervals (Days 0-190, 190-220, 220-433). n varied along the experiment: 36 – 25 – 23 – 13.

In the present study, individuals displayed a typical pattern of growth for lacertids, characterized by rapid growth during the juvenile period (dependent on environmental conditions) and decline with age, with the growth curve approaching an asymptote (Stamps and Andrews, 1992). Growth-rates varied significantly with time and treatment (Fig. 4B, repeated measures ANOVA Treatment \times Time: $F_{4,20} = 4.9$, $P = 0.01$; number of live animals in each sampling period: 13-36). As expected, growth rates were low in the first interval (September 2009 to March 2010), which encompassed the winter hibernation, increased in the period from March to April, and then decreased in the final interval (April to November 2010) when animals were reaching adult size. During March and April 2010, lizards in the Herb+Ins treatment grew significantly faster than Ctr individuals (Duncan's Pos-hoc $P < 0.01$, $n = 23$). The effects of pesticides on lizard growth have received little attention. In *Sceloporus* lizards repeatedly exposed to malathion, no direct effects on growth were observed (Holem et al., 2008). With regard to the lizards in our study, we cannot rule out the possibility that exposure to pesticides could have influenced growth, particularly in light of the increased oxygen requirements observed in field animals from areas exposed to pesticides (Amaral et al., 2012a). However, we can also attribute this increase in growth to an indirect effect of the pesticides on plant cover. The herbicide application decreased plant cover in the enclosures, and we can speculate that prey

items would be more visible to the lizards and/or that the lizards would have increased basking opportunities and may have reached optimal temperatures for hunting more quickly. The insecticide, on the other hand, could increase food availability for lizards by debilitating prey items. Therefore animals could boost their feeding activity and thus increase their growth rate.

Sub-lethal effects

Mean maximum sprint speed (MMS) and maximum sprint speed (MS) were correlated in the two intermediate periods ($P < 0.001$). Therefore, we chose to use only MS in the subsequent analyses. Maximum sprint speed did not vary significantly between treatments and was not affected by recent exposure to pesticides (Repeated Measures ANOVA Treatment x Exposure Timing: $F_{2,20} = 0.09$, $P = 0.91$; Treatment: $F_{2,20} = 2.5$, $P = 0.11$; Exposure Timing: $F_{1,20} = 0.2$, $P = 0.65$, $n = 25$). Ctr lizards achieved slightly higher velocities (March 1.8 ± 0.53 cm s⁻¹; April 1.8 ± 0.98 cm s⁻¹, mean \pm SD), followed by Herb+Ins lizards (March 1.4 ± 0.42 cm s⁻¹; April 1.6 ± 0.45 cm s⁻¹, mean \pm SD) and Herb lizards (March 1.2 ± 0.43 cm s⁻¹; April 1.3 ± 0.39 cm s⁻¹, mean \pm SD). Similar locomotor performance assays were performed using lizards collected from pesticide exposed corn fields (Amaral et al., 2012a), and among those animals we also did not detect any difference in locomotor performance after exposure to a cocktail of herbicides. In light of previous failures to correlate changes in locomotor performance with exposure to carbamate and organophosphorus insecticides (Holem et al., 2006; DuRant et al., 2007), the data presented here and in our previous studies (Amaral et al., 2012a, 2012c), suggest that these kinds of performance assays are not sufficiently sensitive to evaluate the consequences of pesticide exposure.

In our previous field studies we demonstrated increased metabolic demand among individuals sampled from pesticide-treated fields. This was manifested as increased SMR (Amaral et al., 2012a). In the present study, we did not find a similar trend. At the end of the exposure period (5 months after the second pesticide application), lizards exposed to pesticides in mesocosm enclosures had similar SMR to control animals when corrected for body mass (Fig. 5, ANCOVA Treatment: $F_{2,9} = 0.10$, $P = 0.90$, $n = 13$). However, these results might be masked by the number of individuals and by the pooling of genders.

We measured biomarkers of oxidative stress following pesticide exposure (Table 1). Glutathione S-transferase (GST) activity in intestine differed significantly between Ctr lizards and the pesticide treated lizards (ANOVA Treatment: $F_{2,10} = 6.5$, $P = 0.02$, $n = 13$). No differences were found in GST activity in the other tissues. GSTs have an important role in contaminant clearing by conjugating contaminants or their metabolites with glutathione, creating a conjugate metabolite that can be readily excreted. Therefore, we would expect an increase of GST activity in both of our treatments rather than the observed decrease. The mean concentration of reduced glutathione in the intestine of lizards in the Herb treatment was significantly lower than in Ctr individuals (Dunnett's post hoc test: $P < 0.01$). No statistically significant differences were found in the reduced glutathione content of brain and liver or for oxidized glutathione in any of the tissues. We would expect that a reduction in the reduced glutathione concentration would be accompanied by an increase of the oxidized glutathione, which did not occur. Other parameters of oxidative stress such as glutathione reductase (GR) activity and lipid peroxidation showed no signif-

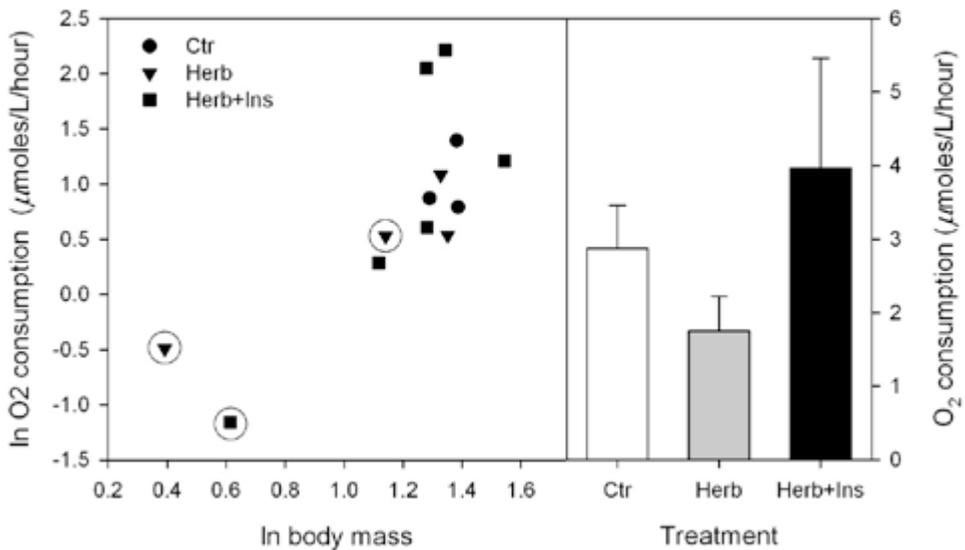


Fig. 5. A. Mean ln-transformed oxygen consumption rates ($\mu\text{moles/L/hour}$) for *Podarcis bocagei* lizards raised in Ctr, Herb and Herb+Ins mesocosms at the end of the exposure period plotted against ln-transformed body mass (g). All measures were taken from post-absorptive lizards at 22 °C ($n = 13$). Encircled points represent females. B. Mean oxygen consumption rates for *P. bocagei* lizards raised in Ctr, Herb and Herb+Ins mesocosm at the end of the exposure period. Bars represent means + standard error ($n = 13$).

ificant variation between Ctr and the two pesticide treatments. Pesticides have been shown to enhance the production of reactive oxygen species and, as a consequence, trigger the molecular and enzymatic mechanisms that counteract oxidative stress. Alterations in glutathiones and/or in the activity of glutathione-related antioxidative enzymes have been regarded as an indication of oxidative stress (Costantini and Verhulst, 2009; Koivula and Eeva, 2010). The fact that none of the other variables measured showed any variation in this study, together with the results obtained in the field study seem to hamper their utility as biomarkers of contaminant exposure in reptiles.

In a previous laboratory study, we found that lizards exposed to realistic oral doses of chlorpyrifos displayed reduced B-esterases activities (Amaral et al., 2012c). Cholinesterases and carboxylesterases are inhibited by organophosphorus and carbamate insecticides, and have been extensively used as biomarkers of pesticide exposure for aquatic and terrestrial organisms (Sanchez-Hernandez, 2007). There was no difference in cholinesterase activities between Ctr lizards and pesticide exposed lizards, irrespective of the tissue type (Table 1). Lizards from both Herb and Herb+Ins treatments showed lower intestinal carboxylesterase (CbE) activity (4-NPV substrate) when compared to the control (Dunnett's post hoc test: $P < 0.05$). There were also differences in intestinal CbE when the α -NA substrate was used, and in liver CbE with both substrates, but these differences were not significant because of the large variability within treatment groups, and likely related to the

Table 1. Mean (\pm standard deviation) activities of glutathione-dependent antioxidant enzymes, glutathione concentrations and activities of esterases in brain, intestine and liver of *Podarcis bocagei* from control (Ctr, n = 3), herbicide treatment (Herb, n = 4), herbicide+insecticide treatment (Herb+Ins, n = 6).

Biomarker	Brain			Intestine			Liver		
	Ctr	Herb	Herb+Ins	Ctr	Herb	Herb+Ins	Ctr	Herb	Herb+Ins
GST (mU mg protein ⁻¹)	18.3 \pm 1.9	19.5 \pm 8.0	35.0 \pm 22.6	68.5 \pm 2.8	37.9 \pm 8.0*	49.7 \pm 16.2*	778.4 \pm 274.5	359.7 \pm 160.8	436.9 \pm 285.5
GR (mU mg protein ⁻¹)	-	-	-	4.4 \pm 1.5	6.0 \pm 2.4	3.6 \pm 1.4	20.9 \pm 10.1	13.3 \pm 9.5	13.3 \pm 5.1
GSH (nmol mg protein ⁻¹)	20.3 \pm 2.3	23.0 \pm 5.5	39.5 \pm 31.2	2.4 \pm 0.3	1.7 \pm 0.3*	2.5 \pm 1.1	18.4 \pm 11.2	11.1 \pm 5.5	13.3 \pm 2.3
GSSG (nmol mg protein ⁻¹)	5.6 \pm 0.9	5.8 \pm 2.1	10.3 \pm 7.8	2.3 \pm 0.5	1.6 \pm 0.02	2.5 \pm 1.1	11.8 \pm 5.5	7.4 \pm 2.4	7.5 \pm 4.0
LPO (nmol TBARs mg protein ⁻¹)	0.4 \pm 0.03	0.5 \pm 0.2	0.8 \pm 0.4	0.5 \pm 0.1	0.5 \pm 0.2	0.4 \pm 0.2	1.5 \pm 0.6	0.8 \pm 0.3	1.1 \pm 0.2
CbE α -Na(mU mg protein ⁻¹)	-	-	-	41.7 \pm 11.2	28.8 \pm 13.2	27.7 \pm 9.4	107.5 \pm 62.3	67.1 \pm 36.1	74.5 \pm 10.4
CbE 4-NPV (U mg protein ⁻¹)	-	-	-	40.3 \pm 8.7	20.3 \pm 12.9*	22.5 \pm 7.3*	82.6 \pm 57.9	51.0 \pm 13.7	45.6 \pm 16.1
AChE (mU mg protein ⁻¹)	53.1 \pm 8.3	51.5 \pm 25.9	51.5 \pm 28.9	-	-	-	-	-	-

GST – glutathione S-transferase; GR – glutathione reductase; GSH – reduced glutathione; GSSG – oxidized glutathione; LPO – lipid peroxidation; TBARs – thiobarbituric acid reactive substances; CbE – Carboxylesterase; AChE – acetylcholinesterase.

*Statistical difference between treated and control animals (Dunnett's post hoc test: P < 0.05).

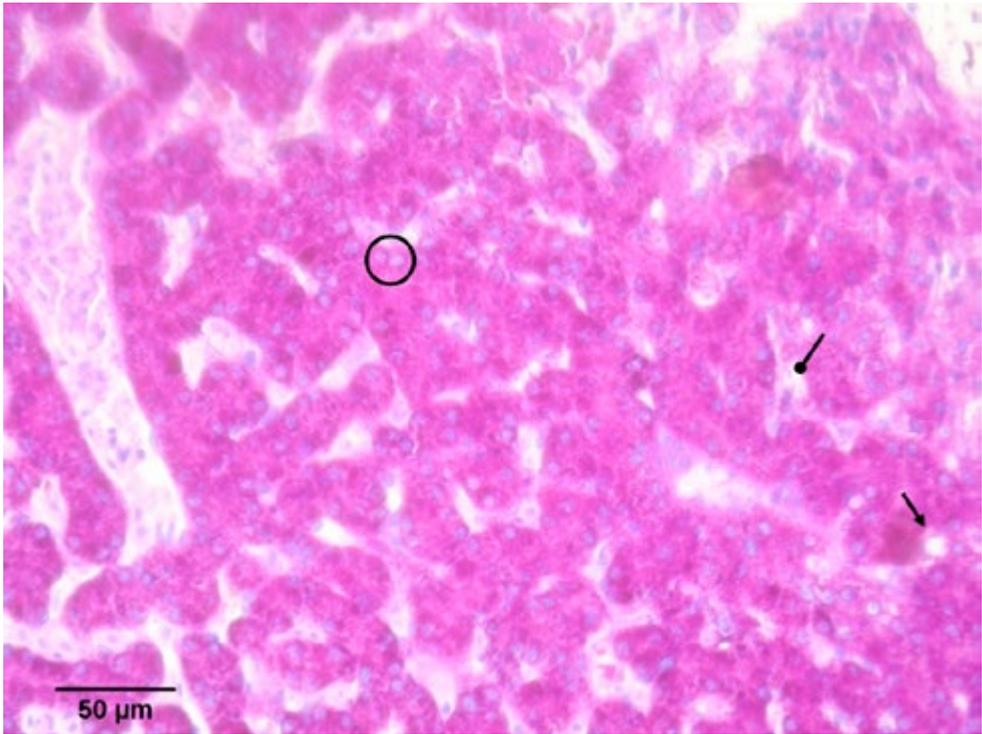


Fig. 6. Representative liver section of *Podarcis bocagei* lizard raised in Herb treatment mesocosm, stained with Periodic acid-Schiff - glycogen vacuoles (PAS positive) are circled; the black arrow indicates round shaped lipid vacuoles and the round-headed black arrow represents other vacuoles.

low number of individuals. CbE are implicated in pesticide detoxification pathways and have been shown to be more sensitive than ChEs in several laboratory and field studies (Wheelock et al., 2008). Sanchez-Hernandez et al. (2009) described higher basal levels of CbE in the anterior intestine of *Lumbricus terrestris* earthworms compared to other tissues, and hypothesized that the alimentary canal could have an important role in pesticide detoxification, thereby reducing the uptake of pesticides. In laboratory experiment with chlorpyrifos, CbE also reacted more strongly than cholinesterases and were strongly inhibited in the gut (Amaral et al., 2012c).

Liver mass did not change between individuals exposed or not exposed to pesticides (ANCOVA Treatment: $F_{2,9} = 1.2$, $P = 0.35$, $n = 13$). There was no statistically significant difference in the occurrence of histological changes in individuals exposed to Herb and Herb+Ins treatments when compared to Ctr (Fisher Exact Tests: $P > 0.33$). However, where those changes were evident, they were manifested as increased prevalence in the treated lizards. In general, individuals from Herb and Herb+Ins treatment groups displayed higher accumulation of iron pigments, severe liver congestion and increased hepatocyte vacuolation and degeneration. These results, despite the lack of significant differences, suggest that pesticide exposed lizards are under stress, and tend to be in agreement

with observations in our previous field study: animals from locations where pesticides were in use, displayed increased hepatocyte degeneration and accumulation of iron pigments (Amaral et al., 2012a). Interestingly, and contrary to what was observed with field collected animals, hepatocyte vacuolation was more evident in animals from both pesticide treated groups. In the field study, increased hepatocyte vacuolation was related with an accumulation of lipids and glycogen (PAS positive) in reference animals (Amaral et al., 2012a). In the case of the mesocosm animals, there were three types of vacuoles, the PAS positive glycogen vacuoles, the perfectly round shaped lipid vacuoles and translucent vacuoles that failed to stain for fat or glycogen (Fig. 6). Hepatocyte vacuolation is not characteristic of any particular disease and can be related with several disturbances, including toxicosis (Myers and McGavin, 2007).

CONCLUSIONS

To our knowledge, this is the first published study that has used a mesocosm approach to examine the effects of agricultural pesticides in a reptile. The approach might be used in the future to validate field observations and provide a bridge between highly controlled laboratory studies and ecological studies. In our study, although there were high rates of mortality, surviving *P. bocagei* lizards that were either exposed or not exposed to pesticides in mesocosm enclosures for over one year thrived and reached adult size. Minor differences in growth rates can be attributed to an indirect effect of the applied pesticides on vegetation coverage and food availability. Our results are in general concordance with those obtained in our previous experiments with animals exposed in field conditions – lizards tend to show some individual symptoms of pesticide exposure, but in general seem able to compensate. However, the low number of individuals that survived in our study prevented robust data comparisons. Therefore, low survival needs to be accounted for in future studies with lacertid lizards. A greater number of individuals in the beginning of the experiment are clearly necessary to compensate for mortality and also to compensate for the fact that sex might influence biomarker results. The main obstacle to achieving large numbers of animals in each mesocosm was the intensive effort required to locating and capturing adequate numbers of gravid females within a relatively short mating season, which would need to be surmounted in future experiments. Despite the difficulties encountered, we believe mesocosms would provide a useful tool for ecotoxicology studies with lacertid lizards.

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REFERENCES

- Alexander, G.J., Horne, D., Hanrahan, S.A. (2002): An evaluation of the effects of deltamethrin on two non-target lizard species in the Karoo, South Africa. *J. Arid Environ.* **50**: 121-133.
- Amaral, M.J., Bicho, R.C., Carretero, M.A. Sanchez-Hernandez, J.C., Faustino, A.M., Soares, A.M., Mann, R.M. (2012a): The use of a lacertid lizard as a model for reptile ecotoxicology studies: part 2 - biomarkers of exposure and toxicity among pesticide exposed lizards. *Chemosphere* **87**: 765-774.
- Amaral, M.J., Carretero, M.A., Soares, A.M.V.M., Mann, R.M. (2012b): The use of a lacertid lizard as a model for reptile ecotoxicology studies - Part 1 Field demographics and morphology. *Chemosphere* **87**: 757-764.
- Amaral, M.J., Sanchez-Hernandez, J.C., Bicho, R.C., Carretero, M.A., Valente, R., Faustino, A.M.R., Soares, A.M.V.M., Mann, R.M. (2012c): Biomarkers of exposure and effect in a lacertid lizard (*Podarcis bocagei* Seoane) exposed to chlorpyrifos. *Environ. Toxicol. Chem.* **31**: 2345-2353.
- Anastassiades, M., Lehotay, S.J., Štajnbaher, D., Schenck, F.J. (2003): Fast and easy multiresidue method employing acetonitrile extraction/partitioning and "dispersive solid-phase extraction" for the determination of pesticide residues in produce. *J. AOAC Int.* **86**: 412-431.
- Bancroft, J.D., Stevens, A. (2002): Theory and practice of histological techniques. Churchill Livingstone, London, UK.
- Banks, J.E., Stark, J.D. (1998): What is ecotoxicology? An ad-hoc grab bag or an interdisciplinary science? *Integr. Biol.* **5**: 195-204.
- Bennet, A.F., Dawson, W.R. (1976): Metabolism. In: *Biology of the Reptilia*, pp. 127-223. Gans, C., Dawson, W.R., Eds, Academic Press, London, UK.
- Boone, M.D., James, S.M. (2005): Aquatic and terrestrial mesocosms in amphibian ecotoxicology. *Appl. Herpetol.* **2**: 231-257.
- Bradford, M.M. (1976): A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* **72**: 248-254.
- Brown, R., Pérez-Mellado, V., Diego-Rasilla, J., Garcia, J., Naranjo, A., Speakman, J. (1992): Individual and population energetics of a lizard on a Mediterranean islet. *Oecologia* **91**: 500-504.
- Bunyan, P.J., Jennings, D.M., Taylor, A. (1968): Organophosphorus poisoning. *J. Agric. Food Chem.* **16**: 326-331.
- Carr, R.L., Chambers, J.E. (1991): Acute effects of the organophosphate paraoxon on schedule-controlled behavior and esterase activity in rats: dose-response relationships. *Pharmacol. Biochem. Behav.* **40**: 929-936.
- Carretero, M.A., Ribeiro, R., Barbosa, D., Sá-Sousa, P., Harris, D.J. (2006): Spermatogenesis in two Iberian *Podarcis* lizards relationships with male traits. *Anim. Biol.* **56**: 1-12.
- Civantos, E., Forsman, A. (2000): Determinants of survival in juvenile *Psammodromus algirus* lizards. *Oecologia* **124**: 64-72.
- Costantini, D., Verhulst, S. (2009): Does high antioxidant capacity indicate low oxidative stress? *Funct. Ecol.* **23**: 506-509.

- DuRant, S.E., Hopkins, W.A., Talent, L.G. (2007): Impaired terrestrial and arboreal locomotor performance in the western fence lizard (*Sceloporus occidentalis*) after exposure to an AChE-inhibiting pesticide. *Environ. Pollut.* **149**: 18-24.
- Ellman, G.L., Courtney, K.D., Andres, V., Jr., Feather-Stone, R.M. (1961): A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* **7**: 88-95.
- Eyer, P., Worek, F., Kiderlen, D., Sinko, G., Stuglin, A., Simeon-Rudolf, V., Reiner, E. (2003): Molar absorption coefficients for the reduced Ellman reagent: reassessment. *Anal. Biochem.* **312**: 224-227.
- Galán, P. (1986): Morfología y distribución del género *Podarcis* Wagler, 1830 (Sauria, Lacertidae) en el noroeste de la Península Ibérica. *Rev. Esp. Herp.* **1**: 85-142.
- Galán, P. (1997): Reproductive ecology of the lacertid lizard *Podarcis bocagei*. *Ecography* **20**: 197-209.
- Galán, P. (1999): Demography and population dynamics of the lacertid lizard *Podarcis bocagei* in north-west Spain. *J. Zool.* **249**: 203-218.
- Galán, P. (2004): Structure of a population of the lizard *Podarcis bocagei* in northwest Spain: variations in age distribution, size distribution and sex ratio. *Anim. Biol.* **54**: 57-75.
- Gaston, K.J., Chown, S.L., Mercer, R.D. (2001): The animal species-body size distribution of Marion Island. *Proc. Natl. Acad. Sci. U. S. A.* **98**: 14493-14496.
- Gevao, B., Semple, K.T., Jones, K.C. (2000): Bound pesticides residues in soils: a review. *Environ. Pollut.* **108**: 3-14.
- Gomori, G., Chessick, R.D. (1953): Esterases and phosphatases of the brain. A histochemical study. *J. Neuropathol. Exp. Neurol.* **12**: 387-396.
- Guirlet, E., Das, K. (2012): Cadmium toxicokinetics and bioaccumulation in turtles: trophic exposure of *Trachemys scripta elegans*. *Ecotoxicology* **21**: 18-26.
- Habig, W.H., Pabst, M.J., Jakoby, W.B. (1974): Glutathione S-Transferases. *J. Biol. Chem.* **249**: 7130-7139.
- Hill, T., Lewicki, P. (2006): *Statistics: Methods and Applications*. StatSoft, Oklahoma, USA.
- Hissin, P.J., Hilf, R. (1976): A fluorometric method for determination of oxidized and reduced glutathione in tissues. *Anal. Biochem.* **74**: 214-226.
- Holem, R.R., Hopkins, W.A., Talent, L.G. (2006): Effect of acute exposure to malathion and lead on sprint performance of the western fence lizard (*Sceloporus occidentalis*). *Arch. Environ. Contam. Toxicol.* **51**: 111-116.
- Holem, R.R., Hopkins, W.A., Talent, L.G. (2008): Effects of repeated exposure to malathion on growth, food consumption, and locomotor performance of the western fence lizard (*Sceloporus occidentalis*). *Environ. Pollut.* **152**: 92-98.
- Koivula, M.J., Eeva, T. (2010): Metal-related oxidative stress in birds. *Environ. Pollut.* **158**: 2359-2370.
- Mackay, D., Shiu, W.A., Ma, K.-C., Lee, S.C. (2006): *Handbook of physical-chemical properties and environmental fate for organic chemicals*. Taylor & Francis, Boca Raton, USA.
- Mann, R.M., Serra, E.A., Soares, A.M.V.M. (2006): Assimilation of cadmium in a European lacertid lizard: is trophic transfer important? *Environ. Toxicol. Chem.* **25**: 3199-3203.

- Marsili, L., Casini, S., Mori, G., Ancora, S., Bianchi, N., D'Agostino, A., Ferraro, M., Fossi, M.C. (2009): The Italian wall lizard (*Podarcis sicula*) as bioindicator of oil field activity. *Sci. Total Environ.* **407**: 3597-3604.
- Mitchelmore, C.L., Rowe, C.L., Place, A.R. (2006): Tools for assessing contaminant exposure and effects in reptiles. In: *Toxicology of Reptiles*, pp. 63-122. Gardner, S.C., Oberdorster, E., Eds, Taylor & Francis Group, Boca Raton, USA.
- Myers, R.K., McGavin, M.D. (2007): Cellular and tissue responses to injury. In: *Pathologic basis of veterinary disease*, pp. 14-15. MacGavin, M.D., Zachary, J.F., Eds, Mosby-Elsevier, St. Louis, USA.
- Odum, E.P. (1984): The mesocosm. *Bioscience* **34**: 558-562.
- Ohkawa, H., Ohishi, N., Yagi, K. (1979): Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem.* **95**: 351-358.
- Pike, D.A., Pizzatto, L., Pike, B.A., Shine, R. (2008): Estimating survival rates of uncatchable animals: the myth of high juvenile mortality in reptiles. *Ecology* **89**: 607-611.
- Racke, K.D., Steele, K.P., Yoder, R.N., Dick, W.A., Avidov, E. (1996): Factors affecting the hydrolytic degradation of chlorpyrifos in soil. *J. Agric. Food Chem.* **44**: 1582-1592.
- Ramos-Martinez, J.I., Bartolomé, T.R., Pernas, R.V. (1983): Purification and properties of glutathione reductase from hepatopancreas of *Mytilus edulis* L. *Comp. Biochem. Physiol. B* **75**: 689-692.
- Rowe, C.L., Dunson, W.A. (1994): The value of simulated pond communities in mesocosm for studies of amphibian ecology and ecotoxicology. *J. Herpetol.* **28**: 346-356.
- Sanchez-Hernandez, J.C. (2007): Ecotoxicological perspectives of B-esterases in the assessment of pesticide contamination. In: *Environmental Pollution: New Research*, pp. 1-45. Plattenberg, R.H., Ed, Nova Science Publishers, New York, USA.
- Sanchez-Hernandez, J.C., Mazzia, C., Capowiez, Y., Rault, M. (2009): Carboxylesterase activity in earthworm gut contents: Potential (eco) toxicological implications. *Comp. Biochem. Physiol. Part C* **150**: 503-511.
- Sette, L.D., da Costa, L.A.M.A., Marsaioli, A.J., Manfio, G.P. (2004): Biodegradation of alachlor by soil streptomycetes. *Appl. Microbiol. Biotechnol.* **64**: 712-717.
- Stamps, J.A., Andrews, R.M. (1992): Estimating asymptotic size using the largest individuals per sample. *Oecologia* **92**: 503-512.
- Van den Brink, P.J., Tarazona, J.V., Solomon, K.R., Knacker, T., Van den Brink, N.W., Brock, T.C.M., Hoogland, J.P. (2005): The use of terrestrial and aquatic microcosms and mesocosms for the ecological risk assessment of veterinary medicinal products. *Environ. Toxicol. Chem.* **24**: 820-829.
- Wheelock, C.E., Phillips, B.M., Anderson, B.S., Miller, J.L., Miller, M.J., Hammock, B.D. (2008): Applications of carboxylesterase activity in environmental monitoring and toxicity identification evaluations (TIEs). *Rev. Environ. Contam. Toxicol.* **195**: 117-178.
- Zar, J.H. (1996): *Biostatistical analysis*. Prentice Hall, Upper Saddle River, USA.