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The use of a lacertid lizard as a model for reptile ecotoxicology studies - Part 1 Field demographics and morphology

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ABSTRACT

At the European level, lacertid lizards have been proposed as potential model species for reptile ecotoxicology. We studied demographic and morphological aspects of natural field subpopulations of *Podarcis bocagei* inhabiting similar agricultural habitats which were either regularly exposed to pesticides, or not. Parameters examined in this study included population size and density, sex ratio, adult body size, fluctuating asymmetry in femoral pores and parasite prevalence. In general, we detected few statistically significant differences between the exposed and reference subpopulations. Although field situations are ecologically complex and factors other than pesticides may be acting, the absence of observable effects on field subpopulations is probably indicative that lizards are coping or compensating for this level of exposure.

1. Introduction

In 2010, 28% of the reptiles evaluated by the International Union for the Conservation of Nature (IUCN) were listed as Critically Endangered (CR), Endangered (EN) or Vulnerable (VU) (IUCN, 2010) and environmental pollution has been recognized as one of the main contributing factors (Gibbons et al., 2000; Lange et al., 2009; Todd et al., 2010). Despite consistent calls for greater emphasis on reptile ecotoxicology research, there is still a lack of knowledge regarding the responses of reptiles to contaminants (Hall, 1980; Hopkins, 2000; Sparling et al., 2010). Pesticides, in view of their biocide activity, scale and volume of application, are the major contaminant of concern for terrestrial environments (Sánchez-Bayo, 2011). Reptiles, as non target organisms, can be directly exposed to pesticides through various routes, including: (a) ingestion of contaminated food; (b) accidental or deliberate ingestion of soil; (c) inhalation; (d) maternal transfer to eggs/young; (e) dermal exposure; (f) absorption by eggs of contaminants from surrounding environments (Lambert, 1997; Rauschenberger et al., 2004; e.g. de Solla et al., 2006; Rich and Talent, 2009; Weir et al., 2010) and indirectly affected as a consequence of diminished food availability or plant cover (Sánchez-Bayo, 2011).

Modern pesticides have, over the years, become more specific and safer for non target organisms. Registration of a pesticide for use in agriculture usually requires the submission of data to a regulatory agency (in the EU, the European Food Safety Authority through the Council Directive EC 1107/2009). These agencies generally require evidence that the chemical is not overly toxic to aquatic fauna, bees, terrestrial mammals and birds. Information on toxicity to reptiles is only required if it is available. As a consequence, hundreds of chemicals are continuously being registered for use in agricultural areas worldwide, and the impact of these chemicals on reptiles remains largely unknown.

In all fields of toxicology, specific species are being adopted to act as surrogate models for field and laboratory based research. Given their prevalence in Europe, particularly in the Mediterranean basin (Arnold and Ovenden, 2002; Carretero, 2004), lacertid lizards were identified as potential model species for reptile ecotoxicology (e.g. Mann et al., 2006; Cardone et al., 2008). Primarily, the major advantage of these taxa is one of abundance; in suitable habitat types, these animals occur at very high densities compared to other vertebrate taxa, for example 10 lizards to 1 mammal (Valverde, 1967). Moreover, adult lacertids are characterized by strong site fidelity, spending their entire lives within small home ranges and are usually generalist predators (Galán, 1999; Carretero, 2004). Therefore, their exposure history can be assumed with some certainty. Also, they have relatively short life spans (3–4 years). Hence, they are expected to provide inference on short term impacts.

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Lacertid lizards are also relatively easy to maintain in captivity. Most importantly, like many small opportunistic lizard species around the world, they have taken advantage of man-made habitats (Graziani et al., 2006) that often allow many species to persist and even thrive in otherwise harsh environments. The existence of comparable habitats that occur over wide geographic regions, such as stone walls in Mediterranean agriculture zones, allows for a comparison between areas where pesticides are applied on a regular basis and areas where no pesticides are in use.

At the population level, the effects of exposure to contaminants have rarely been confirmed, although it is expected that they will influence subpopulations in subtle ways in conjunction with other biotic and abiotic factors like disease, predation, competition, food availability, climatic conditions and habitat quality (Banks and Stark, 1998; Meyers-Schone, 2000). At least two studies have related declines in reptile subpopulations with sublethal contaminant exposures through modifications on population dynamics (Guillette et al., 1994; Shelby and Mendonca, 2001). Moreover, numerous parameters of exposure and effect in subpopulations and individuals have been identified as potentially useful, although very few of these have been applied reliably to reptile subpopulations to document population declines.

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. Responses at higher levels of organization are usually hard to relate with contaminant exposure. Although metrics of exposure at the individual level have been used to predict effects, they have seldom been associated with effects at community or population levels (Rattner et al., 2000). We adopted a tiered approach that examined subpopulations of the lacertid lizard *P. bocagei* living in similar habitats (agricultural stone walls), that were either regularly exposed to pesticides, or not. The study has been separated into two subsets of data: 1. demographics and morphological aspects of natural field subpopulations, the details of which are described here; and 2. biomarkers of exposure and toxicity at physiological, biochemical and cellular levels, which are presented in a companion paper (Amaral et al., 2012).

2. Methods

2.1. Study area and species

Fieldwork was carried out in six localities in the north coastal region of Portugal (Fig. 1a). These sites were all agricultural fields with adjoining non cropped field margins and stone walls that provided habitat and shelter for reptile species (e.g. Fig. 1b). Four sites (Exp 1, Exp 2, Exp 3, Exp 4) were under intensive agriculture (exposed) in Vila do Conde (41°19′N, 8°40′W) and two sites (Ref 1, Ref 2) were under organic agriculture (reference) in Vila Nova de Famalicão (41°26′N, 8°30′W), approximately 20 km further east. The exposed sites were corn fields (*Zea mays* L.) with a winter rotation of ryegrass (*Lolium multiflorum* Lam.), where fertilizers, herbicides and insecticides have been applied routinely for more than 30 years. The two reference sites included a pasture and an organic farming field (several cultures) with no history of chemical application. The organic farming fields were within 500 m of conventional sites that are routinely sprayed, therefore some exposure may occur from drift but it was predicted to be quite low. The sites were characterized through consultation with the *Cooperativa Agrícola de Vila do Conde* regarding agricultural practices in the region and with landowners concerning current and historical land and pesticide usage. In the exposed sites, farmers are mainly using a combination of herbicides: a corn pre seedling herbicide, which is usually complemented by a second application of a post emergence herbicide. A third herbicide is used by some of the farmers to control plants in field margins, while an insecticide application is only carried out when necessary. The products used are usually changed every 2–3 years to avoid weed resistance or because of EU restrictions.

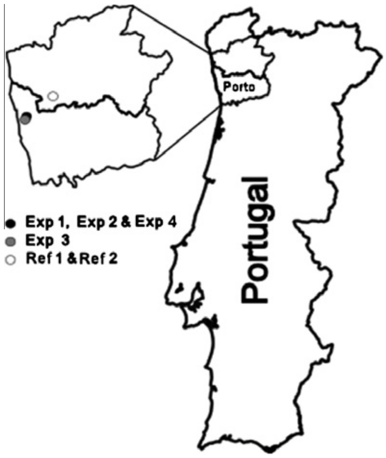

Fig. 1a. Location of the sampling sites on NW Portugal. Exposed sites are shown by a dark or gray circle and the reference areas by a white circle.

Fig. 1b. Example of a sampling site (Exp 2).

The six sites are included in the Temperate Oceanic Sub-Mediterranean climate type with an average temperature <15 °C, a precipitation >1000 mm and a high relative humidity (Rivas-Martinez and Rivas-Saenz, 2009). The fields themselves are not a suitable habitat for lizards and they occupy the adjoining walls and field margins. During the entire field study only one juvenile lizard was observed more than 1 m away from the nearest wall. The margins of the fields were of variable width (from 20 cm to 1 m) and were characterized by the presence of spontaneous herbaceous vegetation.

Podarcis bocagei (Seoane, 1884) is a small insectivorous lacertid endemic to the northwestern Iberian Peninsula. It has a short lifespan (3–4 years) and occupies a variety of habitats, from wide open forests with brushwood to coastal dunes and agricultural areas (Galán, 1986). Adults show a strong sexual dimorphism; males attain larger sizes and a dorsal green coloration whereas females have a brown pattern (Galán, 1986). They are active through most of the year on sunny days, reducing their activity from mid November to February (Carretero et al., 2006). Juveniles hatch between

June and August and reach maturity in 1–2 years (Galán, 2004). Individuals are considered adults if their snout-vent length (SVL) is higher than 47.79 mm for males (Carretero et al., 2006) and 46.11 mm for females (Carretero, unpublished data).

2.2. Soil pesticide concentrations

Soil samples were collected for screening of a list of pesticides and metabolites of present or past use and analyzed by LCMS–MS (Liquid chromatography–tandem mass spectrometry). The screening list included: (1) chemicals currently used in corn fields in the area of study and some of their metabolites (glyphosate, bentazone, dicamba, dimethenamid, alachlor, terbuthylazine, desethylterbuthylazine, mesotrione, chlorpyrifos, linuron); (2) products used in the recent past and their metabolites or which are regarded as persistent (atrazine, desethylatrazine, heptachlor, lindane); (3) other chemicals of concern in the area because of their intensive use, as signaled by the *Direcção-Geral de Agricultura e Desenvolvimento Rural* (2,4-D).

Sampling was performed on two occasions in 2009, before and after spring pesticide application by the farmers. Soil was sampled every 50 m along the walls, on both sides, with at least four replicates per site. The samples were preserved in clean, new 200 mL glass vials, covered with Parafilm® and sent for analysis (Marchwood Scientific Services, Southampton UK). The samples were air dried and ground and a 2 g representative sample was extracted with 20 mL of acetonitrile (containing 1% acetic acid). This was followed by a partitioning step with magnesium sulfate and a subsequent buffering step with sodium acetate. After mixing an aliquot with methanol, the extract was injected into the LCMS–MS (Agilent 6410 Triple Quad LCMS–MS) without any clean up. Standards were prepared in solvents at seven levels with recoveries in the range 70–120% (Level of Detection -0.5 μg).

2.3. Lizard sampling methods

Each of the localities were occupied by a subpopulation as defined by Wells and Richmond (1995). The six subpopulations were sampled during two seasons (spring and autumn) over two consecutive years – 2008 and 2009. Sites were revisited over 3 d in each season to ensure that a high proportion of the individuals were sampled (2 d in the case of subpopulations Exp 2 and Exp 3 in spring 2009). Sampling occurred during all day to ensure that captures were not biased towards either sex or juveniles, which tend to be active at different times of the day (Galán, 2004). Some bias towards the more conspicuous males was expected, but we believe it was uniform across all sites and sampling times. At each visit, individuals were captured by noose or hand, marked individually by toe clipping and with non-toxic marker paint and released, noting gender, reproductive condition, snout-vent length (SVL, to the nearest 0.1 mm), body mass (to the nearest 0.1 g on a Pesola scale) and size class. Animals were visually inspected for tail break and infection by ectoparasites. Digital photographs of the ventral side were taken for analyzing asymmetries in scalation. Where there was ambiguity in determining gender (i.e. subadults and juveniles), sex was confirmed by counting the number of transverse rows of ventral scales; less than 28 in males, more than 28 in females (Kaliontzopoulou et al., 2005). Toe clipping has been considered the most appropriate method for marking small-sized lizards as it is less stressful than the most frequently recommended technique to replace it – microchip implantation (Langkilde and Shine, 2006). Furthermore, toe loss is a common injury in natural subpopulations, and does not affect overall survival (Hudson, 1996). Care was taken so that the longest toe on the hind foot was never amputated and that at least two other toes always remain intact on each foot.

2.4. Data analysis

Population size and density (per 10 m) within each subpopulation was estimated in each sampling period assuming a closed population scenario using the Schnabel or Petersen methodology (Krebs, 1989). For the entire period of the mark-recapture study, the Jolly–Seber open population model was employed (Krebs, 1989). Adult sex ratio was calculated for each subpopulation and a Pearson's Chi-square test determined departure from the expected 1:1 ratio.

For comparison of adult body size, estimated through the snout-vent length, SVL (see Meiri, 2010), two statistics were used: mean body size, and the statistically more robust, mean asymptotic body size, estimated as the 80th percentile (P80) (Brown et al., 1999). Asymptotic body size is a preferred statistic for comparative studies in which both sexes mature at small sizes because it is less affected by proximate factors than mean size (Stamps and Andrews, 1992; Brown et al., 1999). Following Roitberg and Smirina (2006), we compared subpopulations, considering samples of adult males/females collected from the same site in both years and sampling seasons (2008 and 2009; spring and autumn). Mean asymptotic body size was estimated by bootstrapping (100 samples for each sample size). Mean body size and mean asymptotic body size were compared with a Kruskal–Wallis test, with mean SVL/SVL P80 as the dependent variable and sampling site or subpopulation of origin as the independent factor. Body mass was compared through an analysis of covariance (ANCOVA) with sampling site as fixed factor and log SVL as the covariate. Tukey's multiple comparisons Post-hoc tests were employed to test for significant differences when ANCOVA indicated significant differences.

Fluctuating asymmetry (FA) in femoral pores has been widely used with lacertids as a biomarker of environmental stress (López et al., 2002; Crnobrnja-Isailovic et al., 2005). Femoral pores are easy to identify and count and as such are advantageous over other scalation parameters. Fluctuating asymmetry was estimated by triple independent counts of the number of femoral pores on both sides of the body in adults and juveniles and subtracting the counts of the right-hand side from counts of the left-hand side (R–L). Counting was done using photos and always by the same observer to avoid observer bias errors. Analysis of FA followed Palmer (1994); data were inspected for bad raw measurements (defined as errors of the operator during measurement or data introduction); abnormal individuals (individuals that have altered femoral pores because of other conditions); and outliers. Outliers were identified by the Grubb's test statistic (Zar, 1996). Measurement error (ME), the variability between replicate measurements, was estimated and compared to FA by analysis of variance (ANOVA) with sides and individuals as fixed factors. Test for antisymmetry or directional asymmetry was not performed, as *Podarcis* lizards exhibit ideal FA without evidence of any of those conditions (Lima et al., 2008). Differences in absolute values of FA (|FA|) were tested using Levene's test for heterogeneity of variances among subpopulations (Palmer, 1994).

Parasite prevalence was estimated as the percentage of infected lizards per size class and sex (males/females/subadults). Log-linear models were used to analyze simultaneously the effects of site, sex, and season on the presence of ectoparasites in adult lizards. We fitted different models to the data and analyzed the Pearson Chi-square statistics to choose the model that best describes the data.

All statistics were performed in STATISTICA (version 7.0, StatSoft Inc., USA) PC package (Hill and Lewicki, 2007). Assumptions of homogeneity of variance and normality were examined, and when data failed to meet these assumptions, transformations were performed to fit the data to normal distributions or equal variances where possible. Statistical significance was accepted at $\alpha = 0.05$.

3. Results

3.1. Soil pesticide concentrations

Only products that had been recently applied in the fields were detected in our soil analyses, and all were herbicides, including the active substances mesotrione, glyphosate, bentazone, dicamba, dimethenamid-p, alachlor, and terbuthylazine (LOD = 0.5 μg, Fig. 2, Table 1). The following substances were not detected in our samples: desethylterbuthylazine, chlorpyrifos, linuron, atrazine, desethylatrazine, heptachlor, lindane and 2,4-D.

3.2. Population parameters

A total of 417 capture events (86 in site Exp 1, 40 in site Exp 2, 62 in site Exp 3, 73 in site Exp 4, 80 in site Ref 1 and 76 in site Ref 2) including both captures and recaptures were recorded. Of these, 343 were unique individuals. Population size and density for each of the subpopulations in each sampling time was estimated through a closed model approach. No differences were found between exposed and reference subpopulations, but there was a seasonal difference. Subpopulation densities followed a general trend of more animals in autumn than in spring (Fig. 3), with the exception of site Exp 4 in 2009. A reliable population size estimate for the entire period of the study, which would account for changes related with stochastic events, was not achieved. Adult sex ratio (deviations from 1:1), considering animals collected on both years and sampling seasons, was significantly skewed in population Exp 4 and marginally non significant in subpopulations Exp 1 and Exp 3, with more males than females in the three subpopulations (Pearson Chi-square tests, d.f. = 1: Exp 1 $\chi^2 = 2.9$, $p = 0.09$; Exp 2 $\chi^2 = 0.4$, $p = 0.54$; Exp 3 $\chi^2 = 3.5$, $p = 0.06$; Exp 4 $\chi^2 = 6.0$, $p = 0.01$; Ref 1 $\chi^2 = 1.4$, $p = 0.23$; Ref 2 $\chi^2 = 2.0$, $p = 0.15$).

3.3. Body size measurements

Subpopulation Exp 2 was not considered in the analysis of body measurements. This subpopulation had a low sample size (only 34 unique individuals), which impeded the calculation of an accurate statistic for the characteristics under analysis. For male SVL, the two statistics calculated (mean SVL and SVL P80) were highly correlated ($r^2 = 0.87$, t-test = 0.84, $p = 0.46$). For both parameters we detected a significant difference between subpopulations (Kruskal–Wallis mean SVL: $H_{4,140} = 9.7$, $p < 0.05$; SVL P80: $H_{4,500} = 331.3$, $p < 0.001$) (Fig. 4). For mean SVL the post-hoc test could not detect which subpopulations were contributing to the difference, but a visual exploration of the data indicated Exp 3 and Ref 1. For P80 SVL, there were significant differences between all sites. For females, mean SVL and P80 SVL were not so well correlated ($r^2 = 0.70$, t-test = 2.57, $p = 0.08$). We could not detect any significant difference between subpopulations for mean SVL (Kruskal–Wallis $H_{4,91} = 2.9$, $p = 0.58$) but a significant difference was detected for SVL P80 between all subpopulations, except between Exp 1 and Ref 1 (Kruskal–Wallis $H_{4,500} = 265.6$, $p < 0.001$) (Fig. 4).

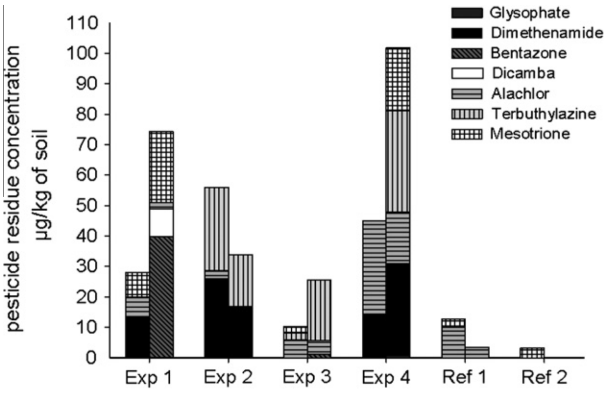

Fig. 2. Average pesticide content of soils (μg/kg soil): seven herbicides were detected (LOD = 0.5 μg/kg) – Pre and post pesticide application in 2009 presented as couplet bars (Exp – Exposed sites, Ref – Reference sites).

For male body mass, a significant difference was detected between the subpopulations (results not shown, ANCOVA Subpopulations: $F_{4,114} = 2.8$, $p = 0.03$). Despite the significance, the pos-hoc test could not discriminate which subpopulations were contributing to the observed difference. Because of the high heterogeneity of variances in female SVL we did not apply the test for female body mass.

3.4. Fluctuating asymmetry

A visual inspection of the scatter plot of left vs right measurements revealed one outlier, which was not considered in the analysis. Between sides variation was significantly greater than measurement error (ANOVA Side × Individual: $F_{1,329} = 14$, $p < 0.001$). The mean number of femoral pores varied between 16.3 and 17.8 and the absolute difference between sides between 0.6 and 0.8 (Table 2). Levene's test for homogeneity of variances did not show any difference between subpopulations or sexes ($F_{11,318} = 1.1$, $p = 0.35$).

3.5. Ectoparasite prevalence

Ectoparasites were all acarids. Ectoparasite prevalence varied between 27% and 63% in females, 7% and 40% in males and 23% and 62% in subadults. Exp 4 lizards had the lowest infection rates. A log linear analysis was performed to distinguish simultaneously between the effects of site, sex and season of sampling on the prevalence of parasites in adults. The model that best described the data indicated that the prevalence of ectoparasites was similar between subpopulations and seasons but that sex significantly affected their presence (Table 3). Females have generally a higher prevalence of ectoparasites than males; in Ref 1 the prevalence was similar.

4. Discussion

Studies that convincingly demonstrate whether environmental contaminants have detrimental effects, or not, on natural reptile subpopulations are still lacking. In agricultural areas in southern Europe, lizards can be considered as valuable environmental indicator organisms, as they are conspicuous and have small home ranges. Exposure to pesticides occurs mostly through ingestion of contaminated food or soil, dermal exposure, and absorption by eggs from surrounding environments.

All products detected on our sites were herbicides that had been used in the past 2 years by farmers. Several other pesticides that were used historically in the region and that would be expected to persist were not detected. For example, atrazine, which was intensively used in corn cultivation until the EU ban in 2007, and its main metabolite (desethylatrazine) were not detected, despite a relatively long residence time in soils (Huber, 1993). In response to concerns for the environment and human health, modern pesticides have, as a generality, 'evolved' to degrade faster in the environment, be more specific in action, and thus, less toxic for non target organisms. This woulpopulations to recover after their disappearance from soil. However, their continuous application in

Table 1
Summary of chemical and ecotoxicological properties of the detected products. Degradation time (NP – non persistent, MP – moderately persistent) Classification of toxicity (? possible; C – carcinogen, ED – endocrine disruptor, RDE – reproductive/developmental effects) (PPDB, 2009).

	Chemical class	Solubility (H_2O 20 °C mg L^{-1})	Volatility (25 °C mPa)	Degradation time (50 d)	Toxic classification
Alachlor	Chloroacetamide	240	2.9	14 (NP)	?C ?ED ?RDE
Bentazone	Benzothiazinone	570	0.17	13 (NP)	–
Dicamba	Benzoic acid	250000	1.67	8 (NP)	?RDE
Dimethenamid-p	Chloroacetamide	1450	2.5	11 (NP)	?C ?RDE
Mesotrione	Triketone	160	5.70×10^{-3}	5 (NP)	–
Terbuthylazine	Triazine	6.6	0.15	75.1 (MP)	?C ?RDE
Glyphosate	Phosphonoglycine	10500	0.0131	12 (NP)	–

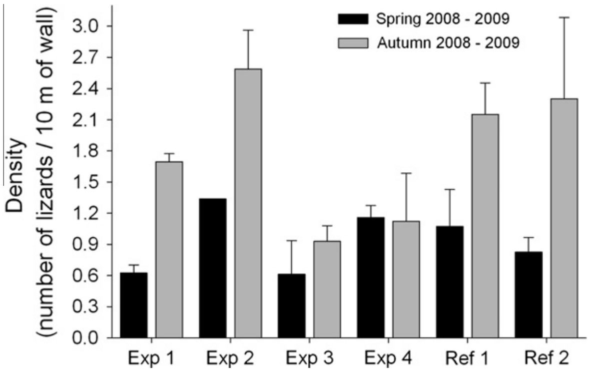

Fig. 3. Mean densities of *Podarcis bocagei* in each site. Each bar represents the mean densities for two collecting seasons (2008 and 2009). Densities are based on either Schnabel or Petersen N estimates (Krebs, 1989). Error bars represent standard deviations ($n = 2$). Absence of an error bar for Exp 2-spring indicates that a density estimate could not be calculated for 2009 (Exp – Exposed sites, Ref – Reference sites).

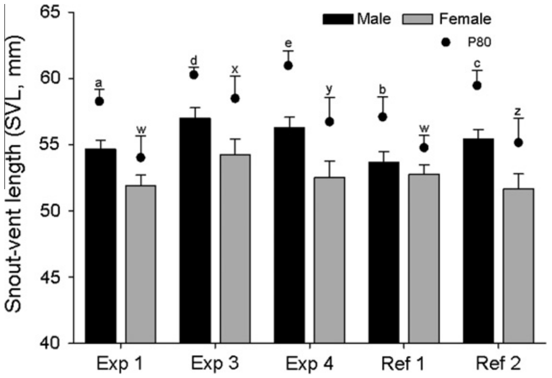

Fig. 4. Mean SVL and SVL P80 of *Podarcis bocagei* adult females and males in the different subpopulations, considering all sampling periods. Error bars represent standard deviations (Exp – Exposed sites, Ref – Reference sites). Distinct letters between sites indicate statistically significant differences (♂ P80 SVL $p < 0.001$; ♀ P80 SVL $p < 0.001$).

agricultural sites and the interaction of the different products used can put subpopulations at risk. As indicated by their presence prior to re-application in 2009, alachlor, terbuthylazine and dimethenamid-p seem to be the most persistent of the chemicals applied, and present themselves as unremitting chemical stressors for terrestrial fauna. Alachlor was detected in one of the reference sites and the source was unknown; it is probably the result of some cross-contamination through air from adjacent fields where pesticides were in use. No data are currently available for the effects of any of these products on reptiles. Data available for birds have classified them as slightly to practically non toxic (PPDB, 2009); however, in a recent survey of literature, reptiles were more sensitive than birds for more than 30% of the chemicals investigated (Weir et al., 2010).

For vertebrate species, it is expected that agriculture intensification will have major effects on abundances within populations rather than on overall biodiversity (Burel et al., 1998). Several studies have found higher levels of activity and density of individuals in organic fields when compared to conventional systems (e.g. Hole et al., 2005; Chamberlain et al., 2010). The majority of these studies have found that the effects of pesticides are mostly indirect, acting through changes in habitat quality related with different management practices. Although the use of pesticides is one of the characteristics of conventional agriculture, no direct correlation with increases in diversity or abundance of mammals or birds was found (Hole et al., 2005; Chamberlain et al., 2010). In the present study, the activity pattern of lizard subpopulations was highly irregular. Despite the high sampling effort, the overall rate of recapture was 15%, which made it difficult to estimate the overarching population sizes for the 2 year study period. Nonetheless, we estimated population size and density for each sampling period, which described an oscillatory trend in lizard abundance over the seasons for each field. Lizard density was higher in autumn than in spring because of juvenile recruitment. In subsequent months high mortality rate among these juveniles have been reported (Galán, 1999, 2004). The subpopulation at Exp 4 was the only population that did not follow this general trend; the autumn population (as proportion of the spring population) was lower. This was also the only subpopulation where the sex ratio was significantly different, with relatively few adult females captured in spring of 2009. Male biased capture rates are generally expected as males are usually more active and visible than females and

Table 2
Mean number of femoral pores in the right (FPR) and left (FPL) side of the body in the individuals of the six subpopulations. Abs. R–L – Absolute value of right minus left number of femoral pores.

Site	*n*	Mean FPR	Mean FPL	Mean FP	Mean abs. (R–L)	Variance abs. (R–L)
Exp 1	74	16.65	16.87	16.76	0.84	0.45
Exp 2	33	17.83	17.89	17.86	0.61	0.35
Exp 3	42	17.13	16.63	16.88	0.74	0.37
Exp 4	61	17.28	17.39	17.34	0.74	0.41
Ref 1	58	16.58	16.55	16.56	0.75	0.61
Ref 2	62	16.32	16.33	16.33	0.79	0.46

Table 3
Results of the log-linear subpopulation prevalence of ectoparasites in *Podarcis bocagei* by season (spring, autumn), age (adults, subadults) and sex (females and males).

Factor	d.f.	Partial Chi-sqr	*p*	Marginal associations Chi-sqr	*p*
Site	5	11.828	0.037	11.828	0.037
Season	1	0.686	0.407	0.686	0.407
Sex	1	3.744	0.053	3.744	0.053
Ectoparasites	1	19.819	<0.001	19.819	<0.001
Site × season	5	7.446	0.190	6.889	0.229
Site × sex	5	3.178	0.673	2.987	0.702
Site × ectoparasites	5	5.503	0.358	5.749	0.331
Season × sex	1	2.113	0.146	1.873	0.171
Season × ectoparasites	1	0.295	0.587	0.492	0.483
Sex × ectoparasites	1	4.063	0.044	4.626	0.031
Site × season × sex	5	4.600	0.467	4.136	0.530
Site × season × ectoparasites	5	3.086	0.687	3.523	0.620
Site × sex × ectoparasites	5	1.750	0.882	1.898	0.863
Season × sex × ectoparasites	1	1.463	0.227	0.767	0.381
Automatic model (site, ectoparasites)		Max. likel.	38.715	41	0.573
		Pearson	36.255	41	0.681
Simplified model (sex-ectoparasites, site, ectoparasites)		Max. likel.	30.345	39	0.838
		Pearson	27.278	39	0.921

the slightly skewed male:female ratio seen in all other sites does not reflect the usual sex ratio in these kind of studies. It is possible that the low number of females in 2009 and consequent low level of juvenile recruitment in 2009 in Exp 4 occurred because of a stochastic event within the population, such as uneven predation, disease affecting only females or greater dispersal of juveniles. However, Exp 4 is also the field with the highest pesticide load, and we can speculate that the reduction in recruitment can be explained by mortality among females and/or lower recruitment in juveniles as a direct or indirect consequence of this exposure.

Demographic studies have been suggested as appropriate tools to assess the total effects of pesticide exposure. Even so, obtaining accurate abundance estimates and relating them with pesticide exposure is not an easy task, because slight variations in demographic parameters can influence population size. None of our study sites represent closed systems, and re-colonization from adjoining subpopulations is likely to be rapid if removal of individuals as a consequence of stochastic (e.g. a harsh winter or autumn) or anthropogenic events (e.g. pesticide exposure) opens up new opportunities for floating colonists (Amo et al., 2007). In our case, most of the adjoining subpopulations occupy similar areas where pesticides are in use; so all subpopulations are under the same type of stress. Nevertheless, population demography in our study cannot be considered as a very reliable or useful parameter to assess pesticide impact. Eventually long-term studies could overcome some of the problems we encountered.

In the absence of clear population indices based on simple abundances of males, females and subadults, it is possible that long-term exposure to pesticides will still be reflected in changes in age structure. For example, lizards might not survive for so long in exposed sites. In a study with the toad *Bufo viridis*, Sinsch et al. (2007) found that age at maturity in males and longevity and post-reproductive life span in females could be related with anthropogenic land use. Additionally, the hypothesis that older individuals were being removed from the population seemed to be the most probable explanation for a difference in population age structure in bullfrogs inhabiting contaminated agricultural sites (Spear et al., 2009). In a parallel skeletochronological study, we distinguished different age structures in the studied subpopulations but we could not detect any clear relationship between herbicide application and a differentiated age structure (Amaral, unpublished data). Alternatively, if energy needs to be re-allocated to detoxification processes as a response to an increase of chemical stressors in their environment, then this might be reflected in maximum body size attained during a lizard's life. Generally, the subpopulations differed in terms of their body size measurements for both females and males. Nevertheless, these differences did not seem to be correlated with herbicide exposure. Changes in size, can reflect differences in growth rates but factors that affect body size have not been well studied. It is considered that body size is affected by processes that differ from those affecting others aspects of animal morphology (Brown et al., 1999). Factors known to influence body size are the size of the mother and conditions during egg incubation (Braña and Ji, 2000). *P. bocagei*, like other oviparous lacertids, deposit their eggs in nests in soil. Egg deposition and incubation occurs during the period when pesticides are being applied in the fields. Exchange of gasses and water with soil during egg incubation can expose them to chemical contaminants. Marco et al. (2004) found that fertilizers had a negative impact on hatchling body length in two *Podarcis* species. We did not study the hatchling parameters in our subpopulations but we can hypothesize that they might be adding noise to our body size results.

Considering that no inbreeding processes are taking place in this population (Pinho et al., 2007), fluctuating asymmetry is another morphological characteristic that can be related with developmental instability during the ontogeny of an individual and ultimately be indicative of population stress (Sarre, 1996) and proximity to pollution sources (Eeva et al., 2000). In our study, no clear differences in FA in femoral pores were found between the subpopulations. Our results are in accordance with another study that found no differences in indices of FA in femoral pores of different subpopulations of the lacertid *Psammodromus algirus* exposed to a heavy metal spill (Márquez-Ferrando, 2008). Other environmental factors, like egg incubation temperature (Zhdanova and Zakharov, 2006) may have stronger effects on FA than pollutants.

In the present study, we also could not detect any differences in parasite presence between the subpopulations. From an ecological perspective, parasite abundance and diversity could be reduced in areas where pesticides are in use (Marcogliese et al., 2005). Alternatively, because exposure to some pesticides is suspected to affect immune responses in some taxa, including amphibians (reviewed in Mann et al., 2009) and mammals (Udoji et al., 2010), it can result in higher infection rates (Carey et al., 1999). None of these scenarios were evident and the only difference observed was related with sex, with females having generally higher prevalences of ectoparasites.

In general, we detected few statistically significant differences between the exposed and reference subpopulations. At least three different scenarios can be proposed to explain the apparent absence of observable effects on population parameters. First, the lack of differences may be because at the concentrations present within the sites these pesticides have no observable effects upon individuals. As indicated earlier, modern day pesticides are increasingly more specific in action, thus reducing the likelihood of effects on non target organisms. Eventually, if more potent pesticides, especially insecticides our results could be different. Second, individuals might have adapted to local stress conditions and therefore survival can be related with acquired natural resistance to contaminants. Third, ultimately, other non toxicological variables can mask pesticide effects.

To fully understand the impact of a contaminant under field conditions it is important to consider also potential impacts of natural stressors. In some of our study sites, other lizard species of larger size that can act both as competitors (when juveniles) and predators (when adults) were also present (*Lacerta schreiberi* and *Timon lepidus*). Predation pressure was probably identical within all sites as several snake and bird species were observed in the sites or close vicinity. The presence of higher level predator species, in particular snakes that have less mobility, might be indicative that contaminant effects are not severe. Also, farming practices varied slightly between fields, and these could be expected to influence habitat quality. Reductions in habitat quality have been related with changes in invertebrate communities and consequently in food availability for vertebrates (Moreby and Southway, 1999). Nevertheless, in a parallel study, we found that even if some of the sites could be more favorable than others to *Podarcis* subpopulations, all fields supported arthropod communities sufficient to sustain these subpopulations (Simão, 2011).

The detectability and activity levels of many reptiles, (highly dependent on survey season), daily timing and environmental conditions have been pinpointed as reasons for the lack of field studies in reptile ecotoxicology (Sánchez-Bayo, 2011). However, the main reasons for this knowledge gap are probably related with the non existence of protocols and model species for this group as there are for other vertebrates. Moreover, the exposure and effects parameters that have been used in the past with other species do not seem the most adequate or applicable to lizard populations in agricultural scenarios. For example, effects in reproductive parameters impacted population dynamics in alligators exposed to endocrine disruptor contaminants (Guillette et al., 2000). However, under agricultural field conditions these parameters are not easily detected for lacertids, as they hide the eggs in soil inside the walls at the margins of the fields. Reliable effect parameters need to be found, especially for field situations.

Field studies should aim to detect correlations rather than effects, as they are not able to explain the complexity of field situations and can only tell us what is happening at a given time and place. Nevertheless, the absence of observable effects on field subpopulations is indicative that lizard subpopulations are able to cope or compensate for this level of exposure. In fact, *P. bocagei* was the most widespread reptile species in this region, surviving in the disturbed agricultural and peri-urban landscape while other species disappeared (Ribeiro, 2011). It can be assumed that this species is either inherently tolerant to the chemicals in their environment or have adapted to the stresses in the agricultural landscape over time. However, considering that there were observable effects at the level of the individual animals, a set of individuals from exposed and reference subpopulations were collected and brought to the lab for measurement of specific biomarker responses. The results of these tests are presented in a companion paper (Amaral et al., 2012).

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