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The impact of land use intensity and associated pesticide applications on fitness and enzymatic activity in reptiles—A field study

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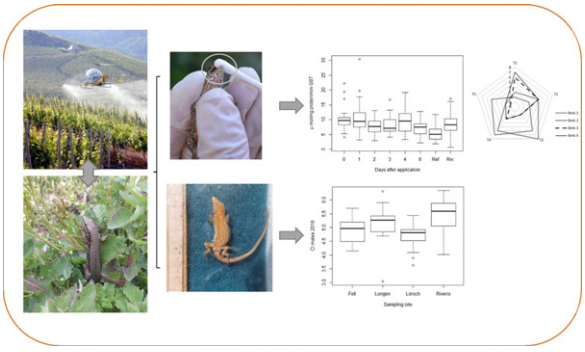
HIGHLIGHTS

- Pesticide exposure caused oxidative stress in lizards inhabiting vineyards.
- Dermal uptake apparently played an important role during the first few days after pesticide exposure.
- Fitness of individuals increased with decreasing land use intensity and pesticide load.
- Age classes in the reference site were higher than in vineyard populations.
- Vineyard populations displayed skewed sex ratios with more male than female lizards.

GRAPHICAL ABSTRACT



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ABSTRACT

Environmental pollution and habitat loss are described as underlying causes for population declines in reptiles and especially affect species in agricultural landscapes. Studies dealing with effects of pesticide exposure on reptiles are limited, mainly addressing the orders Testudines and Crocodylia, but largely neglecting the most diverse reptile order Squamata (lizards and snakes). As a consequence, information regarding effects on their organisms, as well as exposure probability and pesticide uptake in the Reptilia has to be considered rather uncharted. We here ask how pesticide applications affect a widely distributed, synanthropic squamate species in Europe. We studied the common wall lizard (*Podarcis muralis*) with regard to enzymatic biomarkers of pesticide exposure (Glutathione-S-Transferase, Glutathione Reductase, Acetylcholinesterase) and body condition. Lizards were sampled from wild populations, along an exposure gradient (three exposed sites with differing land use intensity and one reference site). Our results suggest both dermal and oral uptake of pesticide formulations, with the former being especially relevant during the first two days after a pesticide application. Enzymatic activity slightly differed between exposure gradients, while showing overall similar patterns. Body condition of lizards decreased with increasing pesticide exposure. Furthermore, gender distribution was particularly skewed in favor to males within exposed sample sites. Although reptiles are not target organisms of pesticide applications, many species do come into contact with them, and most probably suffer from dermal and oral uptake. Thus, we believe it is indispensable for reptiles to be integrated in risk assessments in order to improve conservation practice.

1. Introduction

Environmental pollution is one of the main causes for global biodiversity loss (Benton et al., 2003; Foley et al., 2005; Isenring, 2010;

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Krauss et al., 2010). This also greatly affects reptiles (Gibbons et al., 2000; Todd et al., 2010). According to the IUCN Red List of Threatened Species, these vertebrates remarkably suffer from population declines at the global scale. Out of all reptile species evaluated by the IUCN, 19% are classified as threatened (with 411 species listed in the categories Vulnerable, 382 as Endangered, 196 as Critically Endangered), while in the European Union (EU) alone, 18% of all occurring reptile species are classified as threatened (www.iucnredlist.org; accessed 10.11.16). The underlying causes for reptile declines have been identified as habitat loss and degradation, introduced invasive species, environmental pollution, diseases, unsustainable use and global climate change (Gibbons et al., 2000; Todd et al., 2010). Especially in industrialized countries, the combination of habitat loss and environmental pollution (mainly pesticides) is a significant factor contributing to local and regional biodiversity loss (Gibbons et al., 2000; Todd et al., 2010; Weir et al., 2010). Most habitat loss has historically been caused by agricultural expansion. Although many species have succeeded in adapting and persisting in the altered habitats, they now have to cope with the additional burden of pollution resulting from the increased use of pesticides and other agrochemicals (Gibbs et al., 2009). Many reptiles are characterized by site fidelity and small home ranges. Also, they often show relatively low dispersion capabilities (Böhme, 1981; Huey, 1982; Southwood and Avens, 2010). Thus, these animals' abilities to escape from pesticide exposure are generally hampered. As a matter of fact, recent studies regarding the exposure risk of reptiles to pesticides have shown that at least one third of all species occurring within the EU have an increased exposure risk (Mingo et al., 2016); this even applies to protected areas (Wagner et al., 2015).

Reptiles are non-target organisms of pesticide applications. While relevant for admission procedures (Council Regulation (EC) 1107/2009), they are not integrated during risk assessments due to a lack of guidelines concerning their evaluation. Thus, mammals and birds are commonly used as surrogates (EFSA, 2009; Sparling et al., 2010; Weir et al., 2010). Even worse, little is known about the effects of pesticide formulations on reptiles compared to other vertebrate species (Sparling et al., 2010). A review by Hopkins (2000) revealed that only about 1% of ecotoxicological studies addressing contaminant effects on vertebrate species is focusing on reptiles. Furthermore, there has been a strong unbalance in the studied reptile groups, as most research has focused on the orders Testudines and Crocodylia (Campbell and Campbell, 2002; Hopkins, 2000). However, the majority (94.5%) of all ca. 10,450 reptile species belongs to the order Squamata (i.e. lizards and snakes; Uetz and Hošek, 2016, http://www.reptile-database.org; accessed 10.11.16). As a result, squamates are remarkably under-represented in ecotoxicological studies on the effects of pesticides (Campbell and Campbell, 2002; Hopkins, 2000; Sparling et al., 2010).

While the amount of studies regarding the toxicological effects of pesticides on reptiles are scarce, potentially lethal (Chang et al., 2016; Weir et al., 2015), as well as diverse sub-lethal effects have already been observed. The latter ones encompass a wide array of implications for exposed individuals, ranging from hormonal changes and enzymatic responses, oxidative stress, neurotoxic implications and immunosuppression, to physiological reactions like fever responses, impairments in fertility, development and locomotor performance, over to hermaphroditism (Amaral et al., 2012a, 2012b, 2012c; Bicho et al., 2013; Cardone, 2015; Carpenter et al., 2016; DuRant et al., 2007; Hopkins and Winne, 2006; Schaumburg et al., 2016; Soltanian, 2016; Latorre et al., 2016).

For these reasons, the European Food Safety Authority's (EFSA) pesticide unit is considering the development of a 'Guidance Document' for risk assessment of reptiles (https://www.efsa.europa.eu/sites/default/files/wgamphibian.pdf).

As a means to fill the data gap, in this study, we investigate how pesticide applications affect lizard populations within their natural habitats, by measuring enzymatic activity rates of three well established and suitable biomarkers for pesticide exposure in common wall lizards (*Podarcis muralis*): Glutathione-S-Transferase (GST), Glutathione Reductase (GR) and Acetylcholinesterase (AChE) (Amaral et al., 2012b; Anguiano et al., 2001; Costa et al., 2008; Gavric et al., 2015; Lajmanovich et al., 2011). Additionally, biometric data and gender was gathered for each sampled individual so as to calculate body condition indices (CI), and compare them between surveyed populations. Our objective was to detect how pesticide applications affect enzymatic activities within individuals from three sites with differing land use intensity and pesticide application loads, respectively, as well as with a non-exposed reference site. We expected that GST and GR activities increase following a pesticide application, as these enzymes are responsible for detoxification of xenobiotic substances and combating oxidative stress (Deponte, 2013; Sheehan et al., 2001). On the other hand, AChE activity may decrease due to possible neurotoxic effects resulting from the applied pesticide formulations (Quinn, 1987). Fitness levels of lizards (derived from the CI) from different populations were expected to be affected by the pesticide application loads in the respective sites, as habitat quality between sampling sites was comparable. Finally, we calculated an integrated biomarker response (IBR) for the days following a pesticide application for the exposed sites, in order to better understand the interactions between biomarker activities following pesticide exposure.

Our aims were thus to examine (1) if pesticide exposure induces ecotoxicologically relevant enzymatic biomarker responses within the organisms in their natural habitat. To investigate (2) whether land use intensity and associated pesticide applications have a significant effect on body condition and fitness of individuals along the exposure gradient. To detect (3) possible impacts on population parameters such as gender distribution and age classes.

2. Materials and methods

2.1. Sampling sites and study species

Fieldwork took place at four sites in Rhineland-Palatinate, Germany. Sample sites consisted of three vineyards located in the vicinity of Trier at Lörsch, Longen and Fell; a reference site was located at Riveris. Vineyards were characterized by different land use intensity within and surrounding the sampling site, with an increase of agricultural intensity from Fell, over Longen to Lörsch. The study site at Fell was characterized by 10% agricultural land use (vineyards) within an area of 1 km surrounding it. For the sampling sites in Longen and Lörsch, agricultural land use (vineyards) amounted to 40% and 70% within 1 km of the surrounding area, respectively (Fig. 1). All sites have been used for viniculture for >30 years and are regularly being treated with pesticides in order to control pests (especially fungi) throughout the year. Here, wall lizards especially exploit old dry stone walls as central habitat elements (e.g. for thermoregulation, as hiding place or even as hibernation site) and the surroundings (natural dry vegetation as well as the grape plantations) as foraging habitats (Schulte, 2008).

The reference site, Riveris, was characterized by 0% agricultural land use within 1 km surrounding the area, and is located within a water protection area (Fig. 1). Here, wall lizards use natural rocky outcrops, rocky slopes and dry vegetation as habitat (Schulte, 2008).

The minimum distance between exposed sites was 1 km. The minimum distance to the reference site was 8 km. The majority of applied pesticides were fungicides, which were used from May to August. At one instance, a glyphosate-based herbicide formulation (Clinic Ace®) was sprayed. Applied fungicides during fieldwork were: Vivando®, Polyram WG®, Profiler®, Dynali®, Pergado®, Luna Experience®, Enervin®, Mildicut®, Collis®, Vento Power®, Vegas®, Folpan®, Teldor®, Electis®, Fantic F® (Table 1; for data on the application dates and sampling dates, see Appendix 1). Fungicides were applied in a combination of two to three formulations, in intervals of 7 to 10 days. Applications occurred mainly by aerial dispersion from a helicopter over all exposed sites. The glyphosate-based herbicide Clinic Ace® was applied directly onto the vineyards via ground application. Data

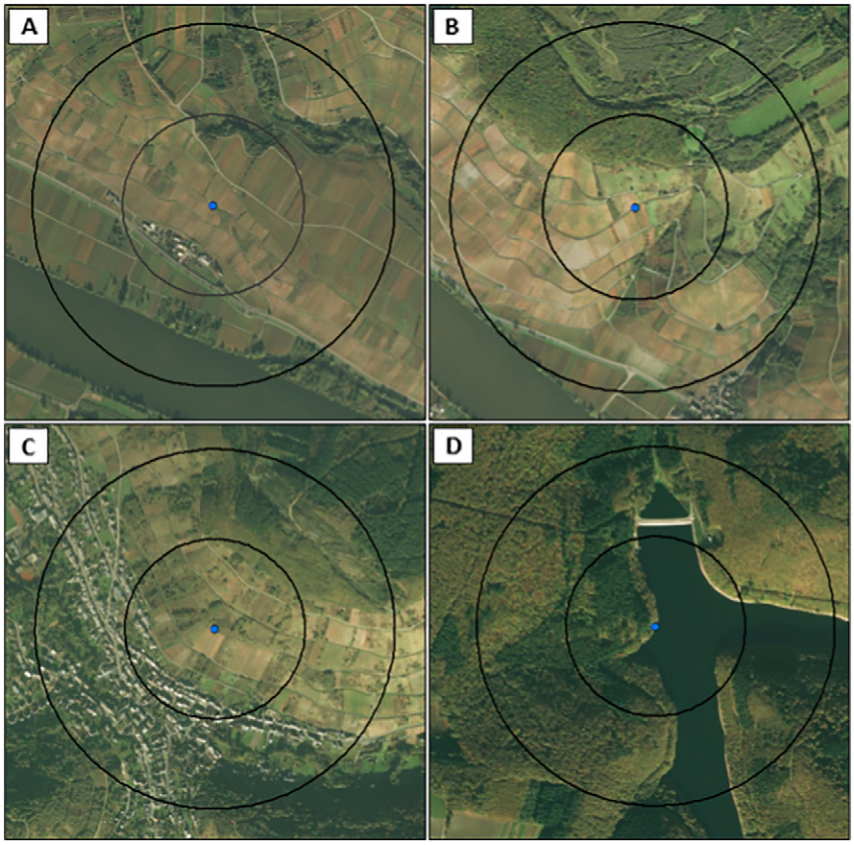

Fig. 1. Surveyed sampling sites, surrounded by a 500 m (inner circle) and 1000 m (outer circle) buffer: (A) Lörsch – 70% agricultural land use within 1000 m; (B) Longen – 40% agricultural land use within 1000 m; (C) Fell – 10% agricultural land use within 1000 m; (D) Riveris – 0% agricultural land use within 1000 m.

on application rates and dates was available thanks to co-operating winemakers.

Podarcis muralis was selected as study species due to its synanthrope character (Schulte, 2008). Although the species shows a predominantly Mediterranean distribution, its natural northern range reaches into southwestern Germany. Here, the species is stenotopic and mainly bound to steep slopes of valleys, which are frequently used for viniculture (Schulte, 2008). Thus, it is strongly associated with agricultural areas and is expected to regularly come into contact with pesticides. This is in particular true as 'grape plantations' show the highest amount of pesticides used by crop within the EU, with >20 kg of active substance/ha (Eurostat, 2007). Therefore, *P. muralis* can be considered a suitable model species to study the effects of pesticide exposure on enzymatic activity rates and fitness levels between natural populations, and to detect potential effects at the individual level. The species mainly occupies adjoining dry stone walls and field margins of vineyards as basking areas, while it also uses the fields themselves as foraging habitat (Böhme, 1986; Schulte, 2008). As a consequence, we expect that uptake of applied pesticide formulations can occur through dermal (e.g. direct over-spraying while basking) and/or oral exposure (ingestion of contaminated food).

2.2. Lizard sampling

Sampling took place throughout the entire activity period of *Podarcis muralis* during the year 2016 (April to September). Individuals were

Table 1
Applied pesticides and application rates (field dose) in the sampling sites during the year 2016.

Pesticide	Active ingredient	Formulation	Type	kg, L/ha
Clinic Ace®	Glyphosate	360 g/L	Herbicide	5
Vivando®	Metrafenone	500 g/L	Fungicide	0.2
Polyram WG®	Metiram	700 g/L	Fungicide	2
Profiler®	Fosetyl-Al & Fluopicolide	667 g/L & 44 g/kg	Fungicide	1.88
Dynali®	Difenoconazole & Cyflufenamid	60 g/L & 30 g/L	Fungicide	0.5
Pergado®	Mandipropamid & Folpet	50 g/kg & 400 g/kg	Fungicide	4
Luna Experience®	Fluopyram & Tebuconazole	200 g/L & 200 g/L	Fungicide	0.5
Enervin®	Initium & Metiram	120 g/L & 440 g/L	Fungicide	3.2
Mildicut®	Cyazofamid	25 g/L	Fungicide	5
Collis®	Boscalid & Kresoxym-methyl	200 g/L & 100 g/L	Fungicide	0.8
Vento Power®	Quinoxyfen & Myclobutanyl	45 g/L & 45 g/L	Fungicide	2
Vegas®	Cyflufenamid	51.3 g/L	Fungicide	0.3
Folpan®	Folpet	800 g/kg	Fungicide	1.6
Teldor®	Fenhexamid	500 g/kg	Fungicide	1.6
Electis®	Mancozeb & Zoxamide	680,5 g/kg & 88 g/kg	Fungicide	1.8
Fantic F®	Folpet & Benalaxyl-M	480 g/kg & 37.5 g/kg	Fungicide	2.4

captured with a noose (Fitzgerald, 2012) while basking. Saliva samples were collected using sterile swabs (Dryswab™, MW113). Buccal swabs have previously been described as a suitable, minimal-invasive method to detect effects of pesticide exposure on enzymatic activity of wall lizards (Mingo et al., 2017). "Conventional" methods to detect potential effects of pesticide exposure were unviable, as sampling would require organ extraction or cardiac puncture (Amaral et al., 2012b; Lajmanovich et al., 2008). Legislation on the protection of animals used for scientific purposes within the EU is strict, even more so for protected species such as the common wall lizard (European Parliament and Council, 2010). Furthermore, the amount of individuals needed to conduct the study was far too great, and would have severely impaired the studied populations. Hence, detecting changes of enzymatic activities in reptiles using buccal swabs has advantages regarding the necessary permissions, the practicability, and ethical aspects.

Swabs were stored on dry ice during fieldwork and later at −80 °C until further processing. Sampling at each site occurred at the beginning of the season (April), before any pesticides had been applied (from 17 April on), and ended on 7 September. The first collected, non-exposed samples were used as control for pristine enzymatic activity rates within the exposed sites. For the analysis of exposed animals, samples were retrieved within seven days after a pesticide application had occurred. A total of 359 individuals were caught, for which buccal swabs were analyzed.

In addition to buccal swabs, biometric data (snout to vent length (SVL) and body mass (BM)), as well as autotomy rates and gender were taken for each captured individual. In order to avoid pseudoreplication, individuals were tagged with waterproof ink. This method allows recognizing already sampled individuals for up to four weeks. Individuals were recaptured whenever sighted in order to renew the tags. Additionally to the individuals caught during 2016, biometric data collected during a previous study (Mingo et al., 2017) were used to calculate condition indices in all sampling sites for the year 2015.

2.3. Enzymatic biomarkers

GSTs comprise a family of phase II metabolic enzymes that catalyze the conjugation of the reduced form of glutathione (GSH) to xenobiotic substrates for the purpose of detoxification (Sheehan et al., 2001). GST activity has often been used as a biomarker for many different contaminants such as insecticides and herbicides (Amaral et al., 2012b; Lajmanovich et al., 2011). It constitutes a standard in vivo biomarker for the exposure to plant protection products as its activity can be altered by a wide range of pesticides.

The function of GR is to catalyze the reduction of glutathione disulfide (GSSG) to the sulfhydryl form GSH, which is critical for resisting oxidative stress and maintaining the reducing environment of the cell (Deponte, 2013). GR has been widely used in multiple studies concerning the exposure of different organisms to pesticides and other xenobiotic substances, and is considered a reliable biomarker to detect oxidative stress including reptiles (Amaral et al., 2012b).

AChE is an enzyme that catalyzes the breakdown of acetylcholine and other choline esters that function as neurotransmitters (Quinn, 1987). Its activity serves to terminate synaptic transmission. AChE is the primary target of inhibition by organophosphorus compounds such as nerve agents and pesticides (Quinn, 1987; Tougu, 2001). AChE has widely been used to assess neurotoxic pesticide effects on organisms (Gavric et al., 2015).

2.4. Enzymatic assays

Frozen buccal swabs were thawed on ice and subsequently homogenized with a Mini-Beadbeater-24 homogenizer (Biospec®). Lysis buffer consisted of 25 mM Tris-HCl and 0.1% Triton X-100. Samples were homogenized for 45 s using 35 mg silica beads for each sample and then centrifuged for 10 min at 10,000 rpm at 4 °C. After centrifugation both steps were repeated. Finally, the supernatant was retrieved and stored at −80 °C until enzymatic analysis started. Protein concentrations were determined by the Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as a standard.

GST activity was determined spectrophotometrically using the method described by Habig et al. (1974). The reaction medium consisted of 150 μL potassium phosphate buffer (100 mM, pH 6.5) and 0.1% Triton-X 100, 20 μL GSH (200 mM), 10 μL 1-chloro-2,4-dinitrobenzene (CDNB, 40 mM) and 20 μL sample. Kinetics was measured using a multi plate reader capable of measuring absorbance at 340 nm. Readings were performed each minute for 10 min, and enzymatic activity was expressed as $\mu mol/mg^{-1}$ protein/min, applying a molar extinction coefficient of 0.00503 μM^{-1}.

GR activity was determined in the manner of Carlberg and Mannervik (1985). The reaction medium consisted of 100 μL potassium phosphate (50 mM, pH 7.5) and 1 mM EDTA, 20 μL GSSG (2 mM), 50 μL NADPH (2 mM) and 20 μL sample. Kinetics was measured using a multi plate reader capable of measuring absorbance at 340 nm. The decrease in absorbance due to NADPH oxidation was measured once every minute for 10 min. Enzymatic activity was expressed as $nmol/mg^{-1}$ protein/min, applying a molar extinction coefficient of 0.00373 μM^{-1}.

AChE activity was measured colorimetrically following Ellman et al. (1961). The reaction medium consisted of 180 μL potassium phosphate (85 mM, pH 7.4) and 0.425 mM 5.5′-dithio-bis(2-nitrobenzoic acid) (DTNB), 10 μL acetylthiocholine (1 mM) and 10 μL sample. Kinetics was measured using a multi plate reader capable of measuring absorbance at 405 nm. Readings were performed once every minute for 10 min. Enzymatic activity was expressed as $\mu mol/mg^{-1}$ protein/min, using a molar extinction coefficient of $1.36 \times 10^4\ M^{-1}\ cm^{-1}$. All assays were performed at 25 °C. All chemicals were obtained from Sigma-Aldrich (Munich, Germany).

2.5. Body condition index

SVL and BM of sampled individuals were used in order to calculate body condition indices (CI). The CI was calculated using the scaled mass index as described by Peig and Green (2009), since it represents an improvement over existing condition indices based on mass and length data (Peig and Green, 2010). According to this method, a bivariate plot of Mass (M) versus Length (L) (in this case, BM vs. SVL) is performed, where the best fit line is obtained by the standardized major axis (SMA) regression on ln-transformed data. Then, the scaled index is calculated for each individual using the following equation:

$$\hat{M}_i = M_i \left[\frac{L_0}{L_i}\right]^{b_{SMA}}$$

The arithmetic mean of SVL was used as value for L_0. M_i–L_i variables represent the raw data for each individual i and b_{SMA} is the slope of the regression. This index adjusts the mass of all individuals to that which they would have at length L_0.

As the study species displays a sexual dimorphism affecting different body proportions between males and females (resulting in generally heavier male lizards; Böhme, 1986, Schulte, 2008), individuals were divided according to gender, in order to better asses the body condition of individuals between sampling sites.

2.6. Integrated biomarker response

The method constitutes a multi-biomarker approach used for in situ assessment of toxicological effects of contaminants, while simultaneously delivering useful data to understand the relationships between biomarkers and contamination levels of studied sites (Beliaeff and Burgeot, 2002; Devin et al., 2014). It is a method that provides both graphical synthesis of the different biomarker responses and a numeric value that integrates all these responses at once. It results from the sum

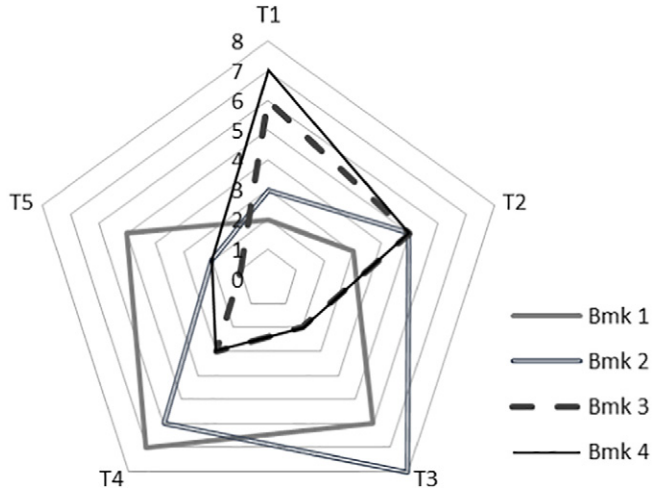

Fig. 2. Example of a star plot used for the calculation of the Integrated biomarker response (IBR, as defined by Beliaeff and Burgeot, 2002), using four biomarkers (Bmk 1 to 4) and five time points (T1 to T5) per sampling site. Each axis of the star plot represents a standardized value (S_i) of a biomarker and two consecutive biomarkers in the plot define a triangle. The sum of the areas (ΣA_i) of the k triangles defines the IBR.

of the area defined by k biomarkers arranged in a radar diagram (Beliaeff and Burgeot, 2002; Fig. 2).

First, the general mean (m) and the standard deviation (s) of all data regarding a given biomarker was calculated, followed by a standardization for each situation to obtain Y, where $Y = (X - m)/s$, and X – in our case – is the mean value for the biomarker during a given day after a pesticide application. Z was then calculated using $Z = -Y$ or $Z = Y$, depending whether a biological effect corresponding to an inhibition or stimulation can be assumed, respectively. For the studied biomarkers, AChE activity was assumed to decrease with increasing pesticide exposure, while the detoxification enzymes GST and GR were considered to be stimulated.

In a subsequent step, the score (S) was calculated following the equation $S = Z + |\mathrm{Min}|$, where $S \geq 0$ and $|\mathrm{Min}|$ is the absolute value for the minimum value of all calculated Y in a given biomarker, for all measurements. Star plots were then used to display Score results (S) and to calculate the IBR as:

$$\mathrm{IBR} = \sum_{i=1}^{n} A_i$$

$$A_i = \frac{S_i}{2} \sin\beta(S_i \cos\beta + S_{i+1} \sin\beta)$$

$$\beta = \arctan\left(\frac{S_{i+1} \sin\alpha}{S_i - S_{i+1} \cos\alpha}\right)$$ (Beliaeff and Burgeot, 2002)

With the goal to calculate the IBR for the overall herbicide and fungicide exposure, as well as days following an application, only enzymatic biomarkers (GST, GR, AChE) were used, as their activity can be expected to be affected by exposure intensity, which varies between all sites. Conversely, the CI was not integrated into the IBR, as it is probably affected by the overall exposure within a site, but not by a specific application date, and will thus not vary in concordance with it. IBR calculations were always performed with the same order of parameters for all sampling sites: first, the detoxification biomarker GST, followed by the oxidative stress biomarker GR and finally the neurotoxicity marker AChE.

2.7. Statistical analyses

All analyses were conducted using *R* (R Developmental Core Team, Vienna). Assumptions of homogeneity of variances and normality distribution of data were examined (using Levene's test and Shapiro-Wilk test). As these assumptions were violated, non-parametric tests were employed to determine significant differences between enzymatic activity rates during sampling days within a sampling site. Since enzymatic activity data for days following a pesticide application are dependent within a study site, Friedman tests were performed in order to test for significant differences. Whenever significant differences could be observed between tested groups, Dunn-Bonferroni tests were run as post-hoc-tests using the 'PMCMR' package in R. As fungicides were applied in a combination of two to three different formulations, there is no way to differentiate between effects of single formulations on enzymatic activity rates. Thus, activity rates for days following a fungicide application were not divided in applications, but evaluated together. As only one day following a herbicide application could be evaluated per site, significant increases in enzymatic activity rates were tested using the related samples Wilcoxon signed rank test.

Comparisons of biometric variables, as well as final CI's between sampling sites were conducted using Kruskal-Wallis-tests, as sampling sites are independent, and parametric assumptions were violated. To calculate the SMA for the scaled mass index, the 'smatr' package was used (Freedman et al., 2007).

3. Results

3.1. Enzymatic activity rates – fungicide applications

Enzymatic activity rates for all studied biomarkers (GST, GR, AChE) and sampling sites are summarized in Fig. 3. A significant increase of GST activity was observed in the sites Lörsch and Longen during days 0, 1, 2, 4 (Friedman test, $p < 0.001$, $df = 7$, $x^2 = 28.59$; Dunn-Bonferroni test, $p < 0.05$) and 0, 1, 4 (Friedman test, $p < 0.001$, $df = 5$, $x^2 = 24.43$; Dun-Bonferroni test, $p < 0.05$), respectively. No significant increase in GST activity was observed for Fell (Friedman test, $p > 0.05$, $df = 4$, $x^2 = 2.13$).

Regarding GR activity in the days after exposure in all sampling sites, a similar trend to that of GST was observed. For Lörsch, it significantly increased during days 3, 4 and 6 after application (Friedman test, $p < 0.05$, $df = 7$, $x^2 = 25.4$; Dunn-Bonferroni test, $p < 0.05$). Although activity rates were above those of the control samples in Longen, no significant increase in activity could be detected (Friedman test, $p > 0.05$, $df = 5$, $x^2 = 8.3$), whereas for Fell, GR activity was significantly higher during days 1 and 2 after application (Friedman test, $p < 0.05$, $df = 4$, $x^2 = 13.92$; Dunn-Bonferroni test, $p < 0.05$). Finally, no significant difference in activity was observed for AChE (Lörsch: Friedman test, $p > 0.05$, $df = 7$, $x^2 = 7$; Longen: Friedman test, $p > 0.05$, $df = 5$, $x^2 = 9.53$; Fell: Friedman test, $p > 0.05$, $df = 4$, $x^2 = 4$).

3.2. Enzymatic activity rates – herbicide application

Enzymatic activity rates regarding the studied biomarkers after exposure to Clinic Ace® had taken place are depicted in Fig. 4. For all sampling sites, a significant increase in GST activity was observed following exposure (Lörsch: related samples Wilcoxon signed rank test, $p < 0.05$, $df = 2$, $x^2 = 9.3$; Longen: related samples Wilcoxon signed rank test, $p < 0.05$, $df = 2$, $x^2 = 6.4$; Fell: related samples Wilcoxon signed rank test, $p < 0.05$, $df = 2$, $x^2 = 44$). Concerning GR activity rates, a significant increase in activity was observed in Lörsch (related samples Wilcoxon signed rank test, $p < 0.05$, $df = 2$, $x^2 = 42$), Longen (related samples Wilcoxon signed rank test, $p < 0.05$, $df = 2$, $x^2 = 117$) and Fell on day 2 after the application took place (related samples Wilcoxon signed rank test, $p < 0.05$, $df = 2$, $x^2 = 48$). As for AChE, no significant effects in activity rates were observed at any sampling site (Lörsch: related samples Wilcoxon signed rank test, $p > 0.05$, $df = 2$, $x^2 = 18$; Longen: related samples Wilcoxon signed rank test, $p > 0.05$, $df = 2$, $x^2 = 10$; Fell: related samples Wilcoxon signed rank test, $p > 0.05$, $df = 2$, $x^2 = 37$).

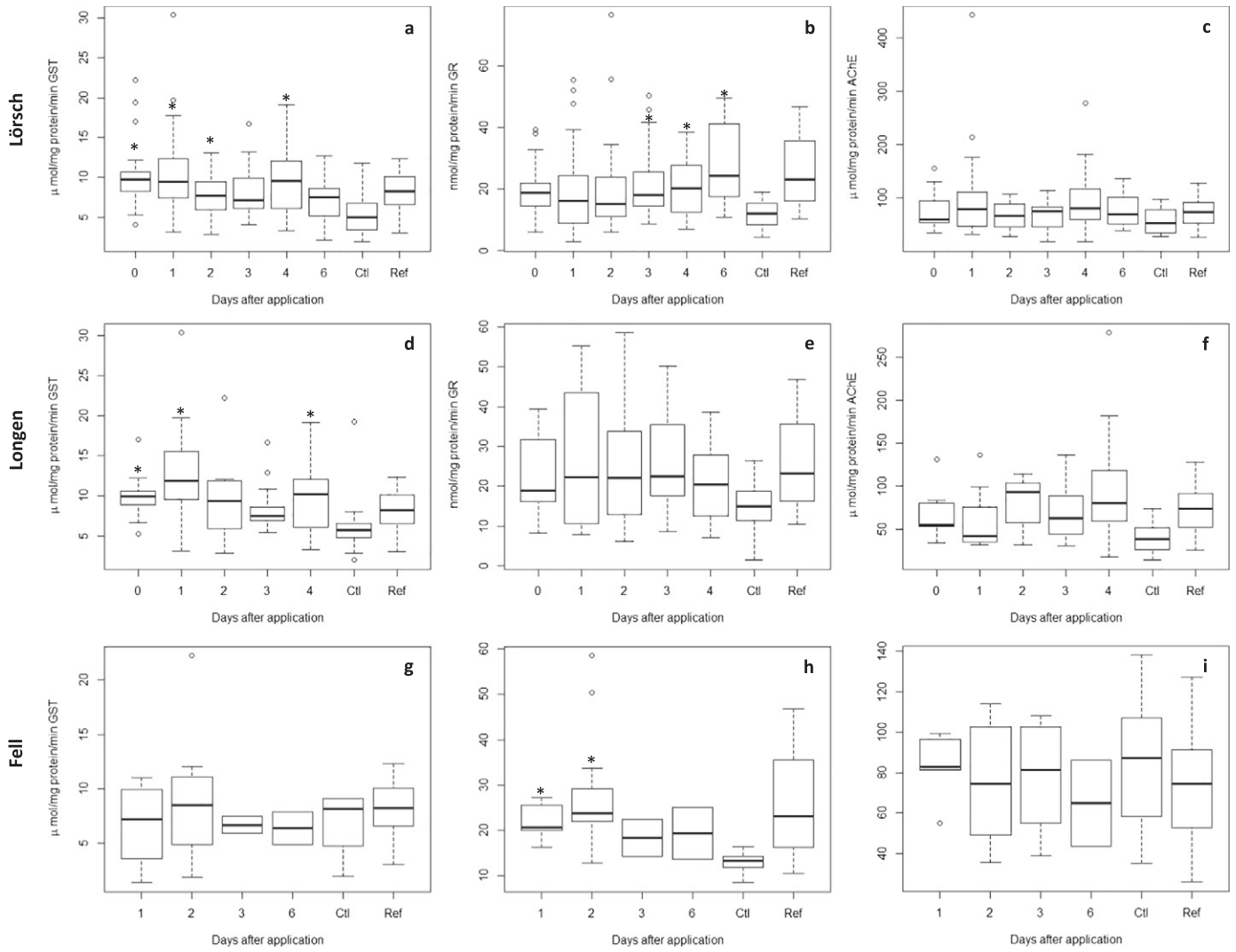

Fig. 3. GST, GR and AChE activity rates for studied individuals exposed to fungicide formulations along the sampling sites: GST activity rates are depicted in sections a, d and g. GR activity rates are represented in sections b, e and h, while AChE is depicted in sections c, f and i. Legend: * – significant difference ($p < 0.05$) in activity rates when compared to control samples; Ctl – Control samples of the respective population (Fell, Longen, Lörsch); Ref – Enzymatic activity measured in the reference site (Riveris).

3.3. Body condition index of sampled individuals

During the year 2015, a slight increase in CI was observed for male individuals sampled from the reference site (Riveris), while the lowest CI was reported for Lörsch. Regarding female lizards caught during the same year, no such effect was observed. In the year 2016, a significant increase of the CI in male individuals of the reference site (Riveris) was observed when compared to two of the exposed sites (Lörsch, Fell; Kruskal-Wallis test, $p < 0.05$, $df = 3$, $x^2 = 14.56$; Nemenyi test, $p < 0.001$ for Lörsch, $p < 0.05$ for Fell). Regarding female individuals captured during 2016, a similar trend in CI was observed, increasing from the most exposed (Lörsch) to the reference site (Riveris). This trend was also reflected in the p value of the Kruskal-Wallis test ($p = 0.08$, $df = 3$, $x^2 = 6.61$).

Concerning the biometric data used to calculate the CI (SVL, BM), a similar trend was noted for males during both years, although more pronounced, displaying the highest BM and SVL for individuals within the reference site, with a general decrease towards increasing exposure/land use intensity. Female specimens showed the same tendency, although variation in biometric data along sampling sites was not as prominent. However, the reference site always displayed the highest values for both parameters (for data regarding SVL, BM and CI's see Appendix 2).

3.4. Autotomy rates and gender distribution along the sampling sites

Autotomy rates were similar in all exposed sites with 30% (Lörsch), 24% (Longen) and 28% (Fell) of individuals showing tail loss. Conversely, autotomy only amounted to 13.7% of sampled individuals in the reference site (Riveris). Concerning the gender distribution, all exposed sites revealed similar patterns, with a male:female ratio of 1.6:1 in Lörsch and 1.76:1 in Fell and Longen. Conversely, the ratio at the Riveris site was 0.78:1.

3.5. Integrated biomarker response

IBR star plots are provided in Fig. 5, IBR scores are presented in Table 2. The IBR conducted for the exposed sampling sites, divided into control, fungicide and herbicide samples, displayed similar trends throughout all sites. The highest enzymatic activity rates were found for the herbicide application, followed by the fungicide applications. When observing the enzymatic activity rates during

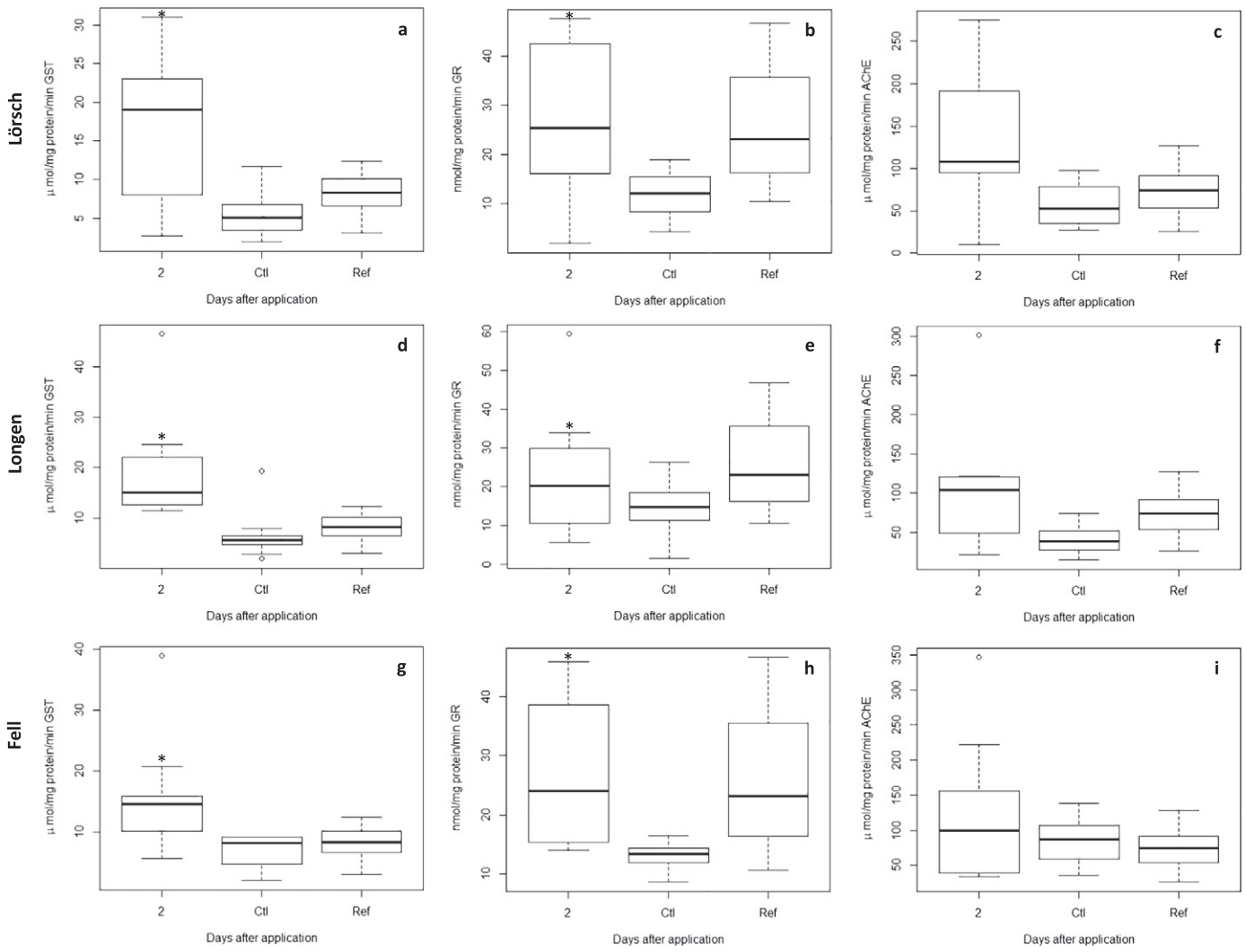

Fig. 4. GST, GR and AChE activity rates for studied individuals exposed to the herbicide formulation Clinic Ace® along the sampling sites: GST activity rates are depicted in sections a, d and g. GR activity rates are represented in sections b, e and h, while AChE is depicted in sections c, f and i. Legend as in Fig. 3.

specific days after a pesticide application, it became evident that the IBR scores increased during each surveyed day after exposure, when compared to control individuals. This was especially the case for the Clinic Ace® application.

4. Discussion

4.1. Enzymatic activity rates

Enzymatic activities of GR and GST observed during the days following pesticide applications are strong indicators towards oxidative stress and detoxification processes, probably caused by the uptake of applied pesticide formulations at the study sites. For AChE, no significant differences in activity could be detected. Consequently, neurotoxic effects of the applied fungicide and herbicide formulations cannot be attested.

The activity patterns for GST indicate an uptake of pesticides both through the dermal and oral pathway in the sampling locations of Longen and Lörsch. This is supported by the observed activity pattern: an initial increase in activity, followed by a normalization of activity and subsequent re-increase. We expect this initial peak to be caused by a dermal uptake of fungicide formulations, as it is plausible that (for instance basking) individuals are becoming over-sprayed via aerial fungicide applications (thus covering a wide, also non-target area; Salyani and Cromwell, 1992). Activity rates subsequently seem to normalize due to ongoing detoxification processes, until oral exposure through food uptake seems to take over, and once again leads to an activity increase (Fig. 3; Sparling et al., 2010, Todd et al., 2010). For instance, these are the two main exposure pathways for reptiles (Hopkins, 2006; Salice and Weir, 2011; Sparling et al., 2010; Todd et al., 2010; Weir et al., 2014).

A delayed effect of oral exposure is plausible, since pesticide concentrations on prey items can be expected to be much lower than direct over-spraying (Knaebe et al., 2006; Pimentel and Levitan, 1986; Pimentel, 1995), consequently needing more time to build up relevant concentrations that significantly affect enzymatic activity. Furthermore, wall lizards – as any reptile – are poikilothermic (Böhme, 1986; Schulte, 2008), thus needing (due to e.g. lower metabolism rates) lower amounts of food when compared to homeothermic vertebrates, for instance birds (Avery, 1978; Nagy et al., 1999).

As a result, it makes sense that oral exposure may take more time to show effects of pesticide uptake on enzymatic activities. This pattern was not observed in Fell in the current study. Given that from the exposed sites, Fell showed the least amount of agricultural land use, this result is also plausible with regard to application area. The likelihood of ingesting contaminated prey is lower, while for actually

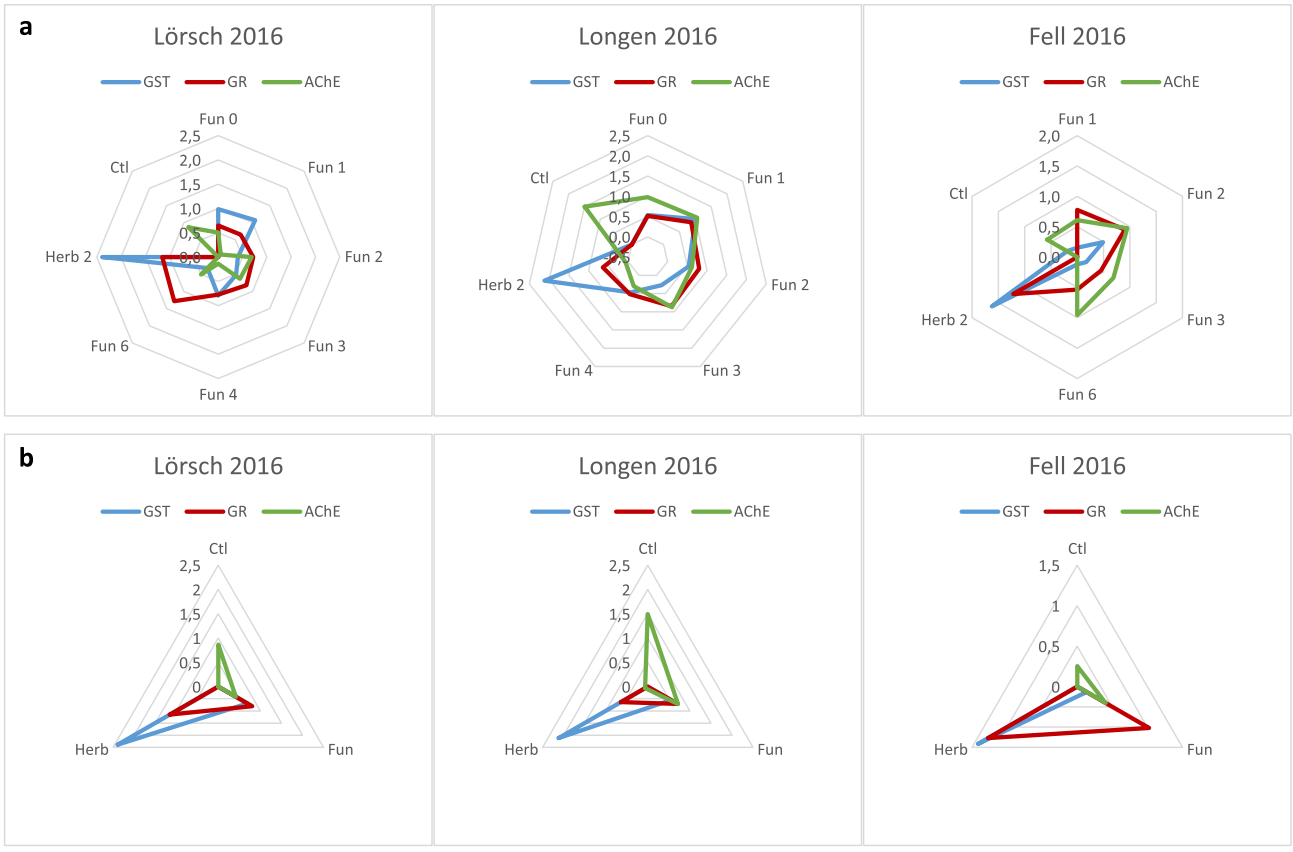

Fig. 5. Star plots used to calculate the IBR for the three exposed sampling sites: Section a depicts specific days following a pesticide application, section b shows the obtained scores for reference individuals, individuals exposed to fungicides and individuals exposed to a herbicide. Legend: GST – Glutathione-S-transferase; GR – Glutathione reductase; AChE – Acetylcholinesterase; Ctl – Control samples from each population; Fun – Fungicides; Fun(X) – Days after a fungicide application; Herb – Herbicide; Herb(X) – Days after a herbicide application.

contaminated prey, intensity ought to be much inferior. Furthermore, individuals within the studied populations cannot be expected to always be exposed to the same pesticide quantities, as intensity varies between different microhabitats (e.g. direct crop land, dry stone walls and fallows; Duelli, 1990, Schulte, 2008, Walklate, 1992).

Regarding GR activity rates, one main finding was that in Lörsch activity increased from day 3 until almost one week after exposure had taken place. The main function of GR is to protect the cells of organisms from oxidative stress and reduce genotoxicity (Deponte, 2013). Consequently, it can be assumed that the applied fungicide formulations are the cause of this stress, which can have severe consequences for individuals (e.g. DNA damage, mutagenic effects, and even suppression of apoptosis and promotion of cell proliferation, invasiveness and metastasis; Halliwell, 2007). Significantly increased GR activity was also observed in Fell on days 1 and 2 after exposure, although not in Longen.

As for the effects of the herbicide Clinic Ace®, application only took place at one instance. Due to bad weather conditions, only one day post application could be examined by us. GST and GR activity rates greatly increased after application in all of the exposed sampling sites, while again, no significant decrease in AChE activity could be observed, and thus, neurotoxic effects could not be detected.

Most interestingly, our results from the year 2016 stand in accordance with our previous study concerning the use of buccal swabs to detect pesticide exposure in wall lizards sampled during the year 2015 (Mingo et al., 2017). Similar trends in activity rates could be observed for GST and GR. The one main difference we found was the evident decrease in AChE activity, observed during 2015 following a glyphosate-based herbicide application (Mingo et al., 2017), which was not detected in the current study. An explanation could be the use of different herbicide formulations (Touchdown® in 2015 versus Clinic Ace® used in 2016). This might lead us to the conclusion that inhibition of AChE was not caused by the active ingredient glyphosate, but rather by the adjuvants used in the Touchdown® formulation (Cox and Surgan, 2006; Wagner et al., 2013), which remain unknown.

Surprisingly, mean enzymatic activities in individuals of the reference site (Riveris) were always slightly higher than for control individuals from exposed sites. Studies have demonstrated that different body parts may be affected differently regarding enzymatic activity rates. While we suggest that saliva is suitable to detect pesticide exposure in lizards (Mingo et al., 2017), we yet do not know how activity relates to blood or internal organs like the liver. In the brown trout (*Salmo trutta*), Almli et al. (2002) have shown that GST activity in gills was significantly inhibited by different fungicide formulations, while no such effect was observed in the liver. A similar effect could be possible for

Table 2
Integrated biomarker response (IBR) for each sampling site and days after application. Legend: Ctl – Control individuals, Fun (X) – Days after a fungicide application, Herb (X) – Days after a herbicide application, Fun* – IBR for all individuals exposed to fungicide formulations, Herb* – IBR for all individuals exposed to herbicide formulations.

Lörsch	IBR score	Longen	IBR Score	Fell	IBR score
Ctl	0.00	Ctl	0.00	Ctl	0.04
Fun 0	0.50	Fun 0	0.45	Fun 1	0.25
Fun 1	0.30	Fun 1	0.89	Fun 2	0.59
Fun 2	0.37	Fun 2	0.44	Fun 3	0.19
Fun 3	0.44	Fun 3	0.42	Fun 6	0.25
Fun 4	0.30	Fun 4	0.20	Herb 2	0.56
Fun 6	0.44	Herb 2	0.54	Fun*	0.22
Herb 2	0.81	Fun*	0.45	Herb*	0.62
Fun*	0.41	Herb*	0.58		
Herb*	1.00				

our lizard saliva samples. Other studies have brought to light that enzymatic activity rates can significantly vary between populations of test organisms in uncontaminated sites (Olsen et al., 2001; Lukkari et al., 2004), or even that activity rates may differ in a gender-biased way (Gallagher et al., 2001; Meyer et al., 1993; Mitchell et al., 1997; Sharma et al., 1993). We therefore strongly recommend the use of control individuals from within the same sample sites when analyzing enzymatic activity rates, in order to avoid natural population variability.

4.2. Integrated biomarker response (IBR)

The star plots of the IBR show an initial increase of GST activity, probably triggered by pesticide exposure as a means of detoxification. As time passes, GR activity starts to increase, indicating that the uptake of pesticide formulations seems to overexert the detoxification capacity of GST, which leads to an imbalance between emerging reactive oxygen species (ROS) and antioxidants, subsequently causing an increase of GR activity in order to reduce oxidative stress (Apel and Hirt, 2004). This trend was especially prominent in Lörsch. Given that this was the most exposed site, it does not come as a surprise, though. The applied fungicides didn't have an inhibitory effect on AChE, as can be seen in the star plots. Generally speaking, the herbicide application triggered the strongest enzymatic reaction in individuals of the exposed sampling sites. Notably, GR activity did not increase as much for the Clinic Ace® application as for the fungicide applications, relative to GST activity. At the same time, oxidative stress was similar for both fungicide and herbicide applications.

When employing the actual IBR scores (Table 2), it was evident that values became worse (higher) during the days following an application, while IBR scores of control samples were always the lowest. In all cases, the highest IBR scores were obtained during the initial two days after exposure to fungicides, indicating a strong effect of pesticide overspray. As for the Clinic Ace® application, the IBR score was in all cases many times higher respective to the control individuals. The IBR made it quite clear that that individuals suffer of greatly increased stress after a pesticide application has taken place, and is evident in all exposed sampling sites.

4.3. Biometric data and condition index (CI)

A clear trend of decreasing BM and SVL with increasing pesticide exposure intensity was observed, which is a strong indicator for generally higher age classes in less exposed but also managed habitats (e.g. plowing and mowing can be also detrimental for wall lizards in the vineyards), as wall lizards keep growing with age (Böhme, 1986; Schulte, 2008). Conversely, it should be kept in mind that body size in reptiles is not always correlated with age (Halliday and Verrell, 1988). Nevertheless, it can be argued that individuals inhabiting sites with a higher pesticide load display a decreased survival rate. This observation is reflected when observing the body condition indices along the sampling sites: the reference site displays a better CI compared to vineyard populations (being especially prominent in male lizards). The trend was observed during both years 2015 and 2016 although it became more evident in the second year. The cause for this decreasing survival and fitness rate can be speculated to be the increasing pesticide load but also other management (like plowing and mowing), resulting in a higher and/or earlier mortality of individuals, as opposed to more remote populations. Other factors like diminished food availability or lack of suitable refuges (Amo et al., 2005; Amo et al., 2007; Ballinger, 1977; Pafilis et al., 2009) can, for the most part, be dismissed, as there was no difference in the availability of hiding places (dry stone walls are even the most prominent habitat elements); conversely, decreased food availability (i.e. prey item abundance) of the different sites was not standardly measured so far. Other environmental variables such as temperature, rainfall or humidity can be neglected here, as all populations stemmed from the same region, with a maximum distance of 12 km between them. Loss of body condition is known to have potentially severe effects on fitness of wall lizards. This includes the capacity to survive hibernation, the ability to compete for breeding opportunities, fecundity and capacity to fight diseases (Amo et al., 2006). Furthermore, it might hamper interspecific competition with sympatric reptile species, such as the sand lizard (*Lacerta agilis*) (Heym et al., 2013). A loss in body condition has further been related to impairments in the immune system of wall lizards (Amo et al., 2006). An increased mortality risk in sites with higher pesticide exposure intensity would consequently be plausible.

With regard to the gender distribution across sampling sites, a skewed male/female ratio could be observed. In the exposed sites, there were in average 1.7 males per female, while in the reference site (Riveris), the sex ratio of an ideal population (0.8 males per female) was observed (Schulte, 2008). Similar results were obtained in a study concerning a closely related species (Bocage's wall lizard, *Podarcis bocagei*) where three of four pesticide-exposed populations displayed a skewed sex ratio, with more male than female lizards (Amaral et al., 2012a). Considering that female individuals displayed generally lower CI's than their male counterparts, it can be hypothesized that, since the trend is a decrease in CI's along the exposure gradient, female lizards in polluted sites will probably be more prone to suffer negative effects from pesticide exposure, possibly increasing the mortality rate respective to male individuals within the same population. Effects like male biased capture rates can probably be overlooked. While in some wall lizard subspecies males are more conspicuous than females, the subspecies known from Germany (*Podarcis muralis merremius*) is not as eye-catching (Böhme, 1986; Schulte, 2008). Coupled with the fact that sampling was always conducted by the same observer (VM), capture bias can be expected to be rather low, and in any case comparable between sampling sites.

Finally, autotomy rates were much higher in exposed sites than in the reference site. While autotomy is apparently not caused by direct pesticide exposure, it is probably a result of the combination of mechanical stress caused by the use of heavy machines (i.e. tractors and other agricultural vehicles). While an important tool for survival, autotomy can be rather prejudicial for lizards, as it can cause locomotor impairments and a reduced hibernation survival, caused by a loss of fat reserves (Brown et al., 1995; Martín and Lopez, 1999). Additionally, tail loss has been associated with a reduced mating success in the Iberian rock lizard (*Iberolacerta monticola*) (Martín and Salvador, 1993), which could, in combination with aforementioned effects, further repress populations persisting in agricultural areas.

4.4. Limits of a field survey

The measured enzymatic activity rates are strong indicators towards detrimental effects of pesticide formulations on reptile wildlife caused by pesticide exposure (namely oxidative stress and genotoxicity). Conversely, it should be noted that a field study ("higher tier testing"; Brown et al., 2009) may not be able to deliver direct cause-relationships between pesticide exposure and enzymatic activity in wild lizards. While this kind of survey has the advantage of generating data which is truthful to natural conditions, it cannot be standardized in such a way as laboratory experiments can be. Possible synergistic or antagonistic effects are much more difficult to detect, and individuals may show additional reactions to other environmental parameters. GST is certainly a good and widely used biomarker for pesticide toxicity, and has been used in multiple studies to test the effects and uptake of different xenobiotics (in many instances pesticides) in different organisms; Anguiano et al., 2001, Lajmanovich et al., 2011, Amaral et al., 2012b). In fact, GST catalyzes the conjugation of GSH to xenobiotic substrates for the purpose of detoxification. It is thus highly unlikely that the observed trends in activity were caused by other environmental parameters (Sheehan et al., 2001). On the contrary, oxidative stress may be induced by other environmental variables than just xenobiotics (e.g. predatory stress; Pinya

et al., 2016). However, individuals were caught while calm and basking, and samples were swiftly taken. We do not believe that individuals were under increased stress before capture, and potential stress caused by capture itself would not generate an immediate oxidative stress response. Furthermore, GR has been widely used to assess potential effects of pesticides on organisms (Costa et al., 2008; Gavric et al., 2015), and showed a similar activity pattern to GST, making the results plausible (as oxidative stress ensues when the detoxification capacity is exceeded). Nevertheless, a final laboratory approach (Tier 1 assessment) is needed to establish a direct cause-relationship between enzyme activities and pesticide exposure. Buccal swabs have been described as a good method to detect effects of pesticide exposure on enzymatic activity of wall lizards (Mingo et al., 2017). Enzymatic data of saliva samples correlated with activity in muscle tissue, and overall, activity patterns in the current study were similar to those already reported in Mingo et al. (2017), thus validating the findings to some extent. However, a final comparison between activity rates in internal organs (e.g. liver) or pesticide residue data is needed, which will be part of future research.

5. Conclusions

Regarding the goals of the study, we were able to verify (1) that exposure to pesticides induced oxidative stress in wall lizards, and can have severe implications for individuals. At the same time, neurotoxic effects were not observed. Moreover, (2) an evident decrease in fitness of individuals was observed with increasing land use intensity (and presumably pesticide exposure). Finally, (3) age groups seemed to follow a similar trend, with higher age classes in less exposed populations, possibly caused by an augmented mortality risk in areas with increasing exposure intensity.

We see that reptiles, in particular of the order Squamata, need to be taken into account for future pesticide admission procedures, as a multitude of negative effects have already been observed, and avian and mammal toxicity data should not be used as surrogate data indefinitely (e.g. Weir et al., 2010), especially given the different lifestyles and biology. We urge the EFSA to include reptiles in future risk assessments, and establish a 'Guidance Document', in order to properly assess the impact plant protection products have on these taxa and improve conservation practice.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2017.02.178.

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