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The use of buccal swabs as a minimal-invasive method for detecting effects of pesticide exposure on enzymatic activity in common wall lizards☆

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ABSTRACT

Habitat loss and environmental pollution are among the main causes responsible for worldwide biodiversity loss. The resulting species and population declines affect all vertebrates including reptiles. Especially in industrialized countries, pollution by agrochemicals is of remarkable importance. Here, habitat loss has historically been associated with expansion of agriculture. Species persisting in such environments do not only need to cope with habitat loss, but more recently, also with chemical intensification, namely pesticide exposure. In this study, we examined effects of different fungicide and herbicide applications on the common wall lizard (*Podarcis muralis*) in grape-growing areas. We used three enzymatic biomarkers (GST, GR, AChE) and for the first time saliva from buccal swabs as a minimal-invasive sampling method for detection. Our results demonstrate absorption of substances by lizards and effects of pesticide exposure on enzymatic activities. Our findings are in accordance with those of previous laboratory studies, although samples were retrieved from natural habitats. We conclude that buccal swabs could become a useful tool for the detection of pesticide exposure in reptiles and have the potential to replace more invasive methods, such as organ extraction or cardiac puncture. This is an important finding, as reptiles are non-target organisms of pesticide applications, and there is a strong need to integrate them into pesticide risk assessments.

1. Introduction

Loss and degradation of habitats, coupled with environmental pollution, is considered a major cause for worldwide biodiversity loss (Benton et al., 2003; Foley et al., 2005; Gibbons et al., 2000; Isenring, 2010; Krauss et al., 2010). The resulting declines of species and populations also greatly affect reptiles. Pesticide usage is suggested to have a dramatic impact on this animal group, especially in industrialized countries (Gibbons et al., 2000; Todd et al., 2010; Weir et al., 2010). Reptiles are non-target organisms of pesticide applications (Sparling et al., 2010), although they often come into contact with them (Mingo et al., 2016; Wagner et al., 2015). Even worse, according to the European Food Safety Authority (EFSA, 2009) reptiles are currently not regarded in pesticide admission procedures, where birds and mammals are used as surrogates. The EFSA pesticide unit is considering the development of the guidance document for risk assessment of reptiles. For that purpose, it is necessary to retrieve more information about the presence and habitat use of these animals in agricultural habitats and to improve the knowledge on their sensitivity to pesticides in comparison to other vertebrates. Along with this, assessment methods need to be tested towards the establishment of standards.

So far, reptiles have been largely neglected when it comes to ecotoxicological research for admission and monitoring of different agrochemicals (including a considerable variety of pesticides; Sparling et al., 2010). In fact, of all ecotoxicological studies concerning pesticide toxicology on vertebrates, reptiles make up only about 1%. At the same time, there is a strong unbalance in the reptile groups examined, as most research in this field has been conducted for the (relatively species-poor) groups of crocodiles and tortoises (orders Crocodylia and Testudines, respectively) (Campbell and Campbell, 2002). However, the majority of all ca. 10,300 reptile species belongs to the order Squamata, i.e. lizards and snakes (Uetz and Hošek, 2016, http://www.reptile-database.org; accessed 25.05.2016). As a result, squamates are especially

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under-represented in ecotoxicological studies (Campbell and Campbell, 2002; Sparling et al., 2010). At the same time, although there has been a comparatively low amount of studies regarding pesticide toxicology in squamates, there are data that indicate lethal effects on exposed individuals at environmentally relevant levels are possible (e.g. Weir et al., 2015). Regarding environmentally relevant concentrations, squamate toxicological studies both under laboratory and field conditions have revealed adverse effects of sublethal pesticide concentrations, such as impairments in fertility of insecticide-exposed Italian wall lizards, *Podarcis sicula* (Cardone, 2015). Likewise, a general loss of body condition, disturbed sex ratios, oxidative stress and an increase of thyroid activity have been observed in Bocage's wall lizards (*P. bocagei*) from the Iberian peninsula after pesticide exposure (Amaral et al., 2012a, 2012b, 2012c; Bicho et al., 2013). Hopkins and Winne (2006) further detected reduction in maximum swimming performance in four colubrid snakes (*Nerodia fasciata, N. taxispilota, N. rhombifer, Seminatrix pygaea*) acutely exposed to high environmental concentrations of the carbamate insecticide carbaryl. Exposure of New Zealand common skinks (*Oligosoma polychroma*) to a glyphosate-based herbicide formulation led to fever responses (Carpenter et al., 2016). It is unknown, however, how these effects may affect entire populations.

The main uptake routes of pesticides for reptiles are suggested to be through dermal and oral exposure, while most attention has generally been given to the latter, being considered the most important exposure route. Dermal exposure has commonly been given less attention, as permeability is considered to be rather low (Hopkins, 2006; Palmer, 2000; Weir et al., 2010). While Weir et al. (2016) recently demonstrated that reptile skin permeability towards pesticides is, in fact, low, a previous study reported that lizards exposed to the same quantities of pesticides via oral and dermal routes resulted in similar residue values (Weir et al., 2014). Thus, dermal uptake should not be disregarded.

In order to assess pesticide exposure of reptiles in their natural habitats, biomarkers are needed, which indicate if individuals do indeed suffer from pesticide uptake. Adequate enzymatic biomarkers for oxidative stress, neurotoxicity and detoxification stress caused by pesticides have already been identified and used to detect pesticide exposure in reptiles, such as Glutathione-S-Transferase (GST), Glutathione Reductase (GR) and different esterases such as Acetylcholinesterase (AChE) (Amaral et al., 2012b; Anguiano et al., 2001; Costa et al., 2008; Gavric et al., 2015; Lajmanovich et al., 2011). The common methods for detecting these biomarkers require invasive procedures (i.e. euthanasia of individuals) such as the removal of internal organs or blood sampling through cardiac puncture (Amaral et al., 2012b; Lajmanovich et al., 2008). This is especially a problem with regard to threatened and protected species. For instance, in the European Union (EU), 18% of all reptile species – that have been evaluated by the IUCN Red List of Threatened Species in 2015 – are considered as threatened, i.e. in the category "Vulnerable" or higher (Cox and Temple, 2009). Simultaneously, legislation on the protection of animals used for scientific purposes within the EU is very strict, even more so for protected species (European Parliament and Council, 2010). Establishing a minimal-invasive sampling method to detect pesticide exposure could thus be of great importance to improve research in this field.

In human pesticide biomonitoring, Henn et al. (2006) have proposed saliva sampling obtained from buccal swabs as a non-invasive method. In lizards, Schulte et al. (2011) have shown that buccal swabbing is a reliable minimal-invasive sampling method for DNA sampling. These observations led us to test this method on wild common wall lizards (*Podarcis muralis*) with regard to enzymatic biomarkers for pesticide exposure and neurotoxicity. Our goal was to test whether the mentioned biomarkers can be measured in reptile saliva, as a means to detect pesticide exposure and uptake into the organism (i.e. increasing or inhibiting enzyme activity after exposure). It can be expected, that detoxification enzyme activities such as GST and GR will increase following a pesticide exposure, while AChE may decrease due to inhibitory effects. In this study, we for the first time employed buccal swabbing on previously used biomarkers (GST, GR, AChE), as a means to create a minimal-invasive method for assessing effects of pesticide exposure on reptiles.

2. Materials and methods

2.1. Sample sites and study species

Sampling and fieldwork took place in three sites in the vicinity of Trier, Rhineland-Palatinate, Germany, during the year 2015. The sample sites consisted of vineyards located near the villages Lörsch, Longen and Fell. The minimum distance between the vineyards was 1 km. All locations have been used for viniculture for more than 30 years, and are regularly being treated with pesticides in order to control pests throughout the year. The majority of applied pesticides were fungicides, which were used from May to August. Fungicides applied during fieldwork were Vivando®, Polyram WG®, Profiler®, Dynali®, Folpan®, Vento Power®, Teldor®, Enervin®, Topas® and Veriphos® (Table 1; for data on the application dates and sampling dates see appendix). Fungicides were applied in a combination of two to three formulations, in intervals of 7–10 days. Applications occurred mainly by aerial dispersion from a helicopter over all sample sites. The glyphosate-based herbicide Touchdown® was applied at one instance during April. This herbicide formulation was applied directly onto the vineyards by ground application. Data on pesticide application rates and dates was made available by co-operating winemakers.

We selected *Podarcis muralis* as study species for pesticide

Table 1
Applied pesticides and application rates (field dose) in the sampling sites during the year 2015.

Pesticide	Active ingredient	Formulation	Type	Kg/ha
Touchdown®	Glyphosate	500 g/l	Herbicide	2
Vivando®	Metrafenone	500 g/l	Fungicide	0,2
Polyram WG®	Metiram	700 g/kg	Fungicide	2
Profiler®	Fosetyl-Al & Fluopicolide	667 g/kg & 44 g/kg	Fungicide	2,81
Dynali®	Difenoconazole & Cyflufenamid	60 g/l & 30 g/l	Fungicide	0,5
Folpan®	Folpet	800 g/kg	Fungicide	2
Vento Power®	Quinoxyfen & Myclobutanil	45 g/l & 45 g/l	Fungicide	2
Teldor®	Fenhexamid	500 g/kg	Fungicide	1,6
Enervin®	Initium & Metiram	120 g/kg & 440 g/kg	Fungicide	3,75
Topas®	Penconazole	200 g/l	Fungicide	0,4
Veriphos®	Potassiumphosphonate	755 g/l	Fungicide	5

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exposure due to its synanthrope character (Schulte, 2008). Although mainly a Mediterranean lizard, its northern distribution range reaches up to southwestern Germany. Here, it is mainly bound to steep slopes of valleys, which are mainly used for viniculture (Schulte, 2008). The species thus is strongly bound to agriculture in its northern distribution range, and is supposed to regularly come into contact with pesticides. Incidentally, the highest amount of pesticides used by crop in the entire European Union (EU) lays within 'grape plantations', with >20 kg of active substance/ha (Eurostat, 2007). Due to its abundance and regular exposure to pesticides within its German distribution area, we considered it to be an ideal candidate species to monitor the effects of pesticide exposure on enzymatic activity and to detect potential effects at the individual level. While the species mainly occupies the adjoining dry stone walls and field margins of vineyards, it does use the fields themselves only occasionally as basking area and foraging habitat (Böhme, 1981; Schulte, 2008). Hence, we do not expect direct over-spraying (dermal absorption) as main uptake of pesticides to common wall lizards but exposure via food, i.e. oversprayed arthropods.

2.2. Lizard sampling

Sampling took place throughout the entire activity period of *Podarcis muralis* during the year 2015 (March–September). Individuals were captured with a noose (Fitzgerald, 2012) while basking on dry stone walls surrounding the vineyards. Saliva samples were then collected using sterile swabs (Copan® 155C). In order to standardize sampling, we let the lizards bite the swab, and slowly rotated it 10 times while in their mouth, avoiding any injuries. Swabs were stored on dry ice during fieldwork and later at −80 °C until further processing. Sampling on each location occurred at the beginning of the season (March), before any pesticides had been applied (from 15th April on), and ended one month after the last pesticide application, which was on 14th August. The first collected, non-exposed, samples were used as reference (control) for non-exposed enzyme activity rates. For the analysis of exposed animals, samples were retrieved within seven days after a pesticide application had occurred, in order to measure biomarker activity rates along a predefined time scale. A total of 245 individuals were caught, for which buccal swabs could be analyzed.

2.3. Studied biomarkers

GSTs comprise a family of eukaryotic and prokaryotic phase II metabolic isozymes known for their ability to 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 been commonly used as a biomarker for many different contaminants such as insecticides and herbicides including reptiles (Amaral et al., 2012b; Lajmanovich et al., 2011). It constitutes a standard *in vivo* biomarker for the exposure to pesticides as its activity can be altered by a wide range of pesticides.

GR catalyzes the reduction of glutathione disulfide (GSSG) to the sulfhydryl form glutathione (GSH), which is a critical molecule in resisting oxidative stress and maintaining the reducing environment of the cell (Deponte, 2013). GR is considered a reliable biomarker to detect oxidative stress produced by pesticide exposure.

AChE is an enzyme that catalyzes the breakdown of acetylcholine and other choline esters that function as neurotransmitters (Quinn, 1987). AChE is mainly found in neuromuscular junctions and in chemical synapses of the cholinergic type, where its activity serves to terminate synaptic transmission. It belongs to carboxylesterase family of enzymes, and 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 were 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 were 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. Enzyme 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 were measured using a multi plate reader capable of measuring absorbance at 405 nm. Readings were performed once every minute for 10 min. Enzyme 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.

Furthermore, for eight wall lizards, additional tissue samples were available as a result of tail autotomy during capture events. Muscle tissue was extracted from the tail base and processed by the same method as mentioned above. In contrast to saliva samples, tissue had to be diluted in a 1:10 ratio before kinetic measurement.

All chemicals were obtained from Sigma-Aldrich (Munich, Germany).

2.5. Statistical analysis

All analyses were conducted with *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 enzyme activity rates during sampling days. Since enzyme 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-

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Bonferroni tests were run as post-hoc-tests to potentially identify them. Correlations between enzyme activity rates were determined according to the Pearson's correlation coefficient, while correlation rates between enzyme activities in different tissue samples were calculated using Spearman's rank correlation, as data violated parametric assumptions. For tissue and saliva samples, linear regressions were additionally calculated in order to determine the amount of variance explained by each model (Freedman et al., 2007).

3. Results

Enzymatic assays for the tested biomarkers using buccal swabs showed a success rate of around 90%.

3.1. GST activity

Fig. 1 (a, d, g) summarizes the mean activities of GST for individuals exposed to fungicide formulations at all three sampling sites. An increase of activity after exposure could be observed through all sampling locations. Days 2 and 3 showed significant increases in activity for Lörsch (Friedman test, $x^2 = 20.78$, $df = 3$, $p < 0.001$; Dunn-Bonferroni test for days 2 and 3, $p < 0.05$). For Longen, GST activity during day 4 after application was significantly higher than for reference samples (Friedman test, $x^2 = 10.9$, $df = 2$, $p < 0.01$; Dunn-Bonferroni test, $p < 0.05$), while the same could be observed in Fell during days 1 and 4 (Friedman test, $x^2 = 12.6$, $df = 3$, $p < 0.01$; Dunn-Bonferroni test, $p < 0.05$).

Fig. 2 (a, d, g) summarizes the mean GST activities for lizards exposed to Touchdown® in all sampling sites. Except for the sampling location in Longen, no significant differences in activity rates could be observed between reference and exposed saliva samples (Longen: Friedman test, $x^2 = 28$, $df = 1$, $p < 0.05$; Dunn-Bonferroni test, $p < 0.05$/Lörsch: Friedman test, $x^2 = 0.4$, $df = 2$, $p > 0.05$/Fell: Friedman test, $x^2 = 12$, $df = 1$, $p > 0.05$).

3.2. GR activity

Fig. 1 (b, e, h) shows the mean activities of GR for studied individuals exposed to fungicide formulations in all three sampling sites. The activity pattern was similar to the one reported for GST, although significant effects on activity rates were only observed in Lörsch, during day 3 after exposure (Friedman test, $x^2 = 8.9$, $df = 3$, $p < 0.05$; Dunn-Bonferroni test, $p < 0.05$ for day 3). For sites Longen and Fell, no significant differences were found when compared to reference samples (Longen: Friedman test, $x^2 = 2$, $df = 2$, $p > 0.05$/Fell: Friedman test, $x^2 = 3.6$, $df = 3$, $p > 0.05$).

Regarding the Touchdown® application, again, no significant differences in enzyme activity rates could be observed for Lörsch (Friedman test, $x^2 = 15$, $df = 2$, $p > 0.05$) and Longen (Friedman test, $x^2 = 21$, $df = 1$, $p > 0.05$). However, Fell showed a significant increase of activity at day 7 after application (Friedman test, $x^2 = 15$, $df = 1$, $p < 0.05$; Dunn-Bonferroni test, $p < 0.05$) (Fig. 2 b, e, h).

3.3. AChE activity

Fig. 1 (c, f, i) provides information on the mean AChE activities for examined lizards exposed to the applied fungicide formulations, in all sampling sites. Fluctuation in AChE activity levels between exposed and reference samples were observed, although no significant effects could be observed for any sampling site (Lörsch: Friedman test, $x^2 = 0.60$, $df = 3$, $p > 0.05$/Longen: Friedman test, $x^2 = 4.67$, $df = 2$, $p > 0.05$/Fell: Friedman test, $x^2 = 4.92$, $df = 3$, $p > 0.05$).

For the samples collected after the Touchdown® application, a reduction of activity rates can be observed for this biomarker, although results were not significant (Lörsch: Friedman test, $x^2 = 1.6$, $df = 2$, $p > 0.05$/Longen: Friedman test, $x^2 = 4$, $df = 1$, $p > 0.05$/Fell: Friedman test, $x^2 = 2$, $df = 1$, $p > 0.05$) (Fig. 2 c, f, i).

3.4. Correlations between enzyme activities

We examined whether correlations existed between enzyme activities for the target biomarkers. A positive correlation was found between GST and GR activities over all samples, as well as after the distinction between fungicide and herbicide exposures (Pearson correlation, all: $p < 0.001$, $df = 155$, $r = 0.32$ (see Fig. 3a) fungicides only: $p < 0.001$, $df = 129$, $r = 0.33$; herbicide only: $p < 0.05$, $df = 62$, $r = 0.30$), while no correlation at all could be observed between GST and AChE activity rates (Pearson correlation, $p > 0.05$, $df = 174$, $r = 0.0023$; Fig. 3b). Between GR and AChE, a negative correlation was identified over all samples (Pearson correlation, $p < 0.05$, $df = 148$, $r = -0.16$; Fig. 3c).

3.5. Correlations between saliva and tail tissue samples

According to a Spearman rank correlation test, GST activities from buccal swabs positively correlated with tissue activity rates taken from muscle tissue ($p < 0.05$, $rho = 0.61$; Fig. 4a), while AChE activity rates did not ($p > 0.05$, $rho = 0.22$; Fig. 4b). For GR, again a positive correlation could be shown ($p < 0.05$, $rho = 0.46$; Fig. 4c). Furthermore, linear regressions showed that for GST, 51% of the variance could be explained by the model ($p < 0.05$, $r^2 = 0.51$), while for GR, 52% of the variance was explained by the model ($p < 0.05$, $r^2 = 0.52$).

4. Discussion

4.1. Enzymatic activities after pesticide exposure

The results of this study indicate pesticide uptake in *Podarcis muralis*, a squamate reptile species, in their natural habitats after exposure to plant protection products. However, it is unclear if only the active ingredient(s), only the adjuvants or the entire pesticide formulation was taken up and which substances were mainly responsible for effects. Further studies on the bioaccumulation and toxicogenetics of the substances in reptiles are necessary to answer this question. An increase of GST activity was observed during the first four days after exposure to different fungicide formulations, in all sampling sites. As GST conjugates GSH to xenobiotic substrates – in this case the pesticide formulations – as a means of detoxification (Sheehan et al., 2001), this increase in activity when compared to non-exposed samples is a strong indicator of detoxification stress by the exposed lizards. GR activity displayed the same pattern as GST for individuals exposed to fungicide formulations (in fact, activity rates between both biomarkers correlated), although activity rates for the former were substantially lower. For GR, a significant increase in activity (when compared to reference samples) could only be observed for the sampling locality in Lörsch, at three days after exposure. The main function of GR is to protect the cells of organisms from oxidative stress and thus reduce genotoxicity (Deponte, 2013). The increase in GR activity observed here, can thus be seen as an indicator for emerging reactive oxygen species (oxidative stress). This oxidative stress can cause direct damage to the DNA, and is expected to be mutagenic, while it may also suppress apoptosis and promote proliferation, invasiveness and metastasis (Halliwell, 2007). As to why this increase in GR activity was only significant for the locality of Lörsch, it may be explained by the varying land use intensity along the sampling sites. In Lörsch, agricultural land use (i.e. vineyards) amounted to 70% of the area

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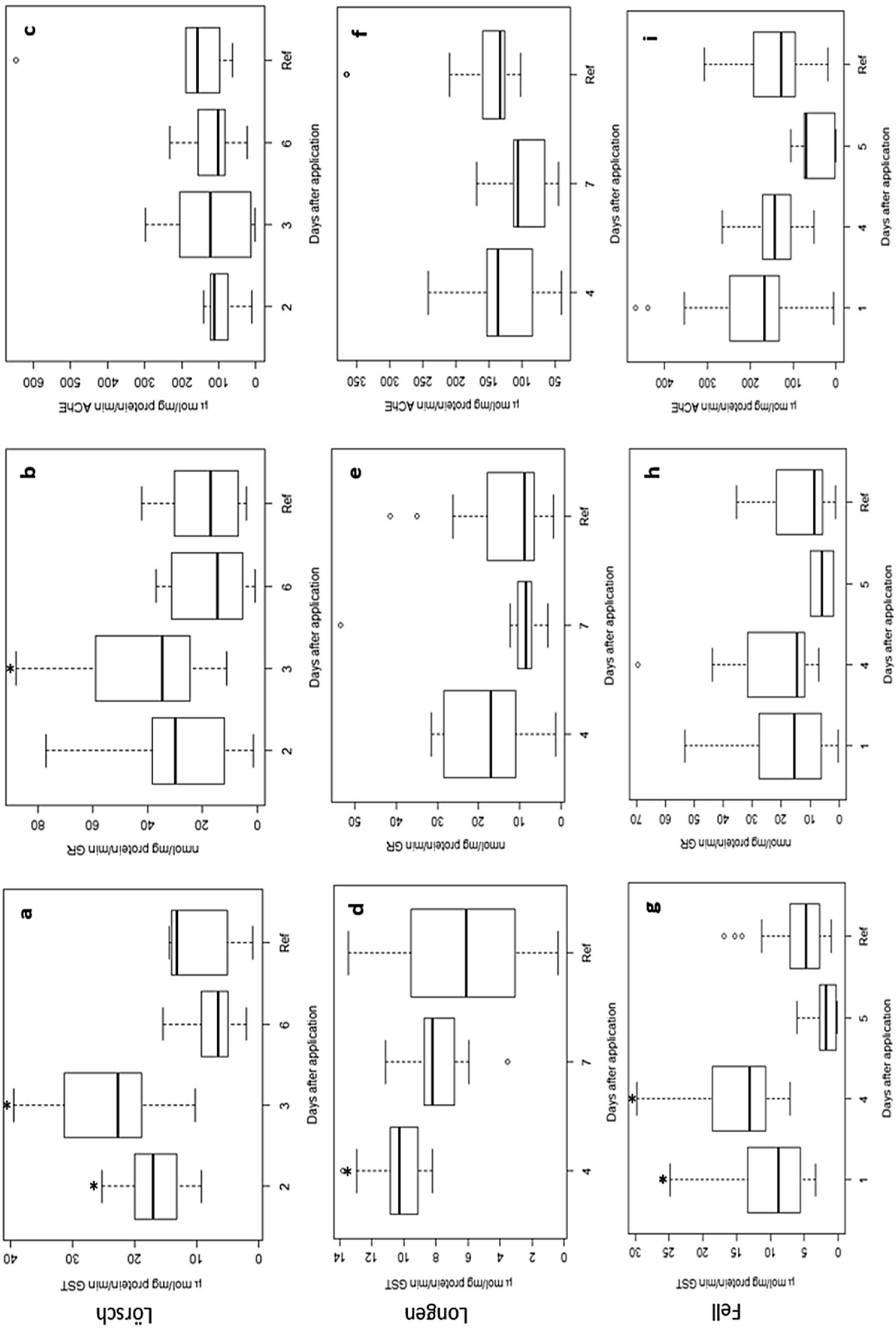

Fig. 1. GST, GR and AChE activity rates for studied individuals exposed to fungicides along the three sampling sites. GST activity rates are depicted in sections **a**, **d** and **g**, GR activity rates are represented in sections **b**, **e** and **h**, while AChE is depicted in sections **c**, **f** and **i**. * - significant difference in activity rates when compared to reference samples. Days 2 and 3 showed significant increases in GST activity for Lörsch (Friedman test, $x^2 = 20.78$, $df = 3$, $p < 0.001$; Dunn-Bonferroni test for days 2 and 3, $p < 0.05$). For Longen, GST activity during day 4 after application was significantly higher than for reference samples (Friedman test, $x^2 = 10.9$, $df = 2$, $p < 0.01$; Dunn-Bonferroni test, $p < 0.05$). The same could be observed in Fell during days 1 and 4 (Friedman test, $x^2 = 12.6$, $df = 3$, $p < 0.01$; Dunn-Bonferroni test, $p < 0.05$). A significant increase in GR activity was observed at day 3 after application for lizards sampled in Lörsch (Friedman test, $x^2 = 8.9$, $df = 3$, $p < 0.05$; Dunn-Bonferroni test, $p < 0.05$).

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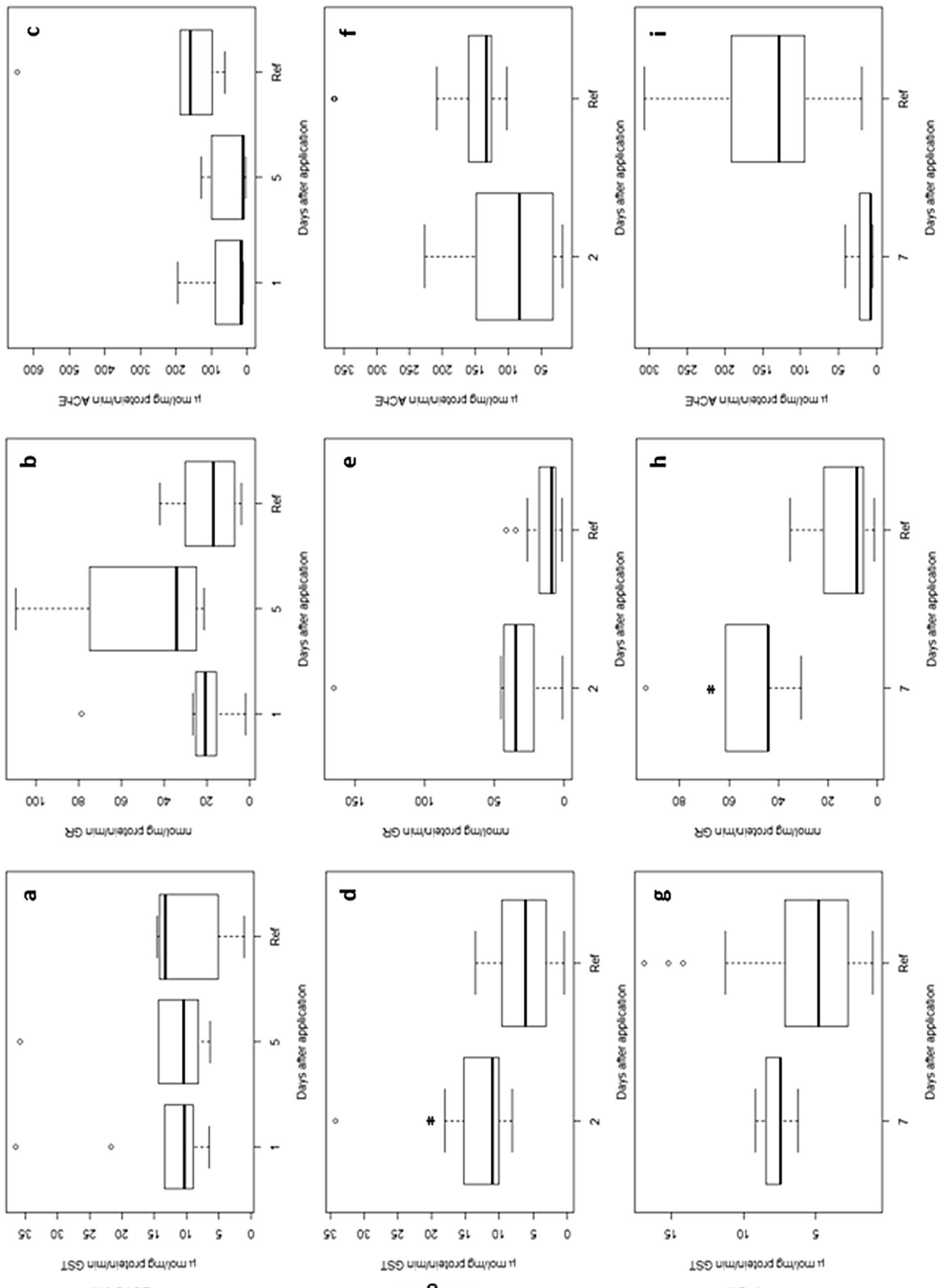

Fig. 2. GST, GR and AChE activity rates for studied individuals exposed to the herbicide Touchdown® along the three sampling sites. GST activity rates are depicted in sections **a**, **d** and **g**. GR activity rates are represented in sections **b**, **e** and **h**, while AChE is depicted in sections **c**, **f** and **i**. Abbreviations as in Fig. 1. Longen showed a significant increase in GST activity at day 2 after exposure to Touchdown® (Friedman test, $X^2 = 28$, $df = 1$, $p < 0.05$; Dunn-Bonferroni test, $p < 0.05$). Furthermore, a significant increase of GR activity was observed in Fell at 7 days after exposure (Friedman test, $X^2 = 15$, $df = 1$, $p < 0.05$; Dunn-Bonferroni test, $p < 0.05$).

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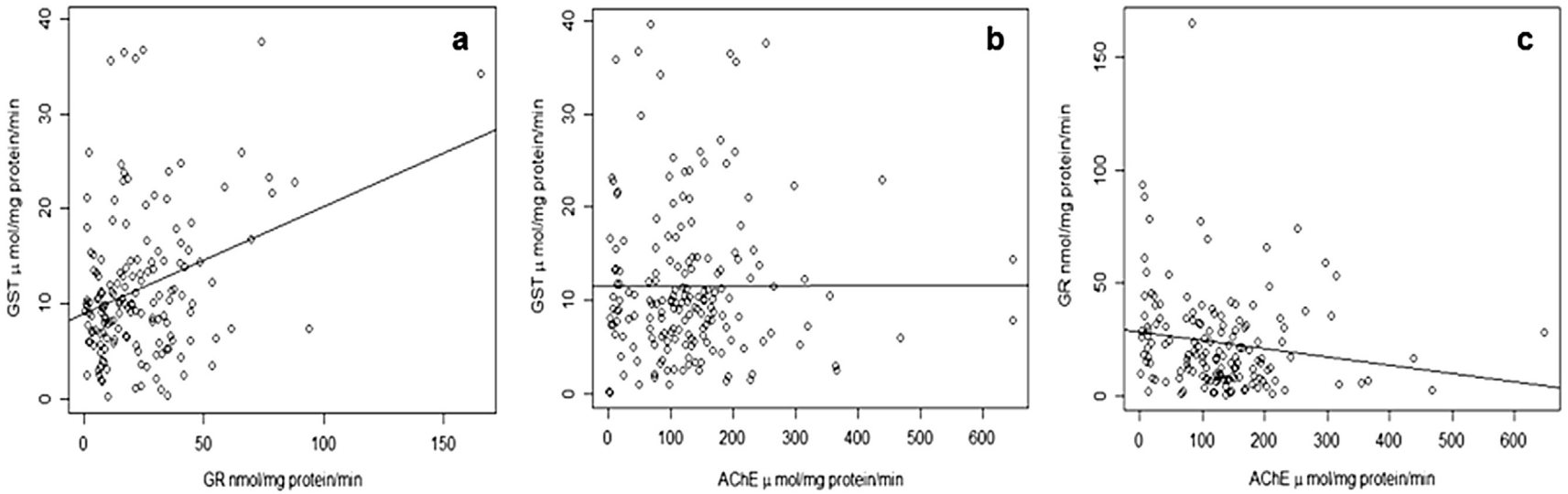

Fig. 3. Scatterplot showing correlations between **a)** GST and GR activity rates ($p < 0.001$, $df = 155$, $r = 0.32$), **b)** GST and AChE activity rates ($p > 0.05$, $df = 174$, $r = 0.0023$) and **c)** GR and AChE activity rates ($p < 0.05$, $df = 148$, $r = -0.16$) for all saliva samples.

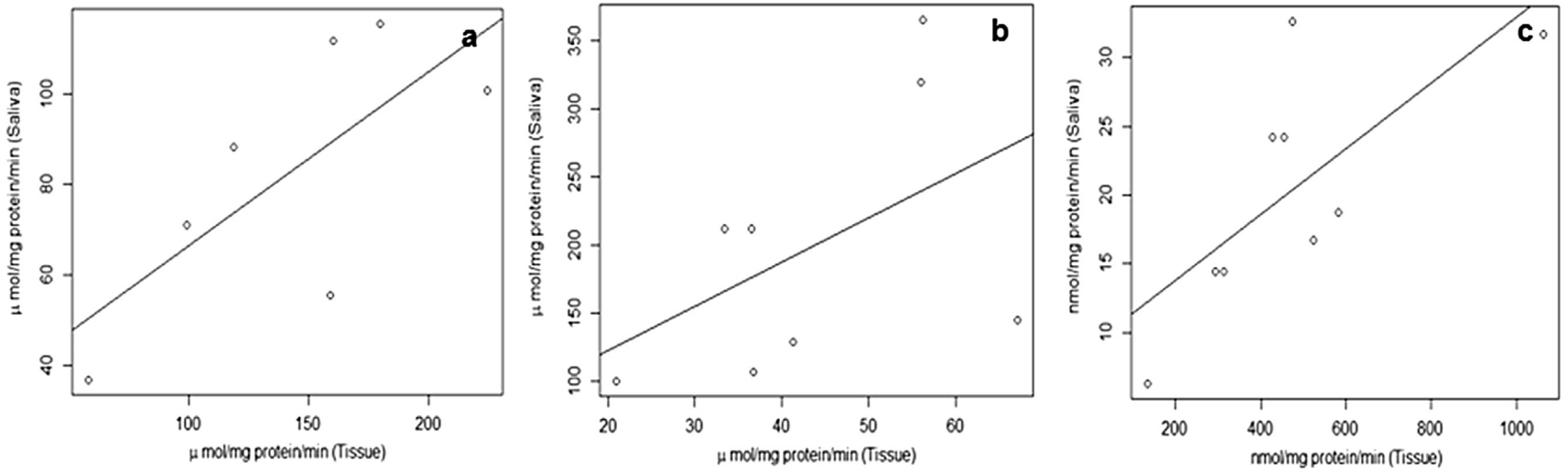

Fig. 4. Scatterplots showing activity in tissue and saliva samples of the studied individuals for **a)** GST ($p < 0.05$, *rho* = 0.61), **b)** AChE ($p > 0.05$, *rho* = 0.22) and **c)** GR ($p < 0.05$, *rho* = 0.46).

within a 1 km buffer surrounding the sampling site. For Longen and Fell, agricultural land use (vineyards again) only amounted to 40% and 10% of the area within 1 km buffers, respectively. Furthermore, specimens of wall lizards and likely of other syntopic squamate species (in our localities smooth snakes, *Coronella austriaca*, sand lizards, *Lacerta agilis*, and slow worms, *Anguis fragilis*) will never be permanently exposed to the same concentrations, as exposure intensity commonly varies between the exploited microhabitats, such as direct crop land, dry stone walls and fallows (Walklate, 1992). Additionally, depending on the areas and microhabitats used for hunting, prey items expectedly exhibit lower or higher contamination levels (Duelli, 1990; Schulte, 2008; Walklate, 1992). It can thus be argued that lizards occurring in areas surrounded by stronger land use intensity will have a higher probability of pesticide uptake, as the odds of coming into contact with the used formulations will increase. This, combined with the much lower activity rates measured for GR (nmol as opposed to μmol for GST and AChE) could be an explanation why the increase in activity was only observed in one site (which was incidentally the one with the highest proportion of agricultural land use). At the same time, it has to be noted that until day 4 after application, GR activity rates were generally higher than in reference samples, in all sampling sites.

It can further be argued that the increase of activity rate may peak at around day three after exposure, followed by a subsequent activity normalization. Such a typical peak has for example been observed when quantifying concentrations of pesticides in herbivore arthropods (Knaebe et al., 2006). A relation between enzyme activity and pesticide residue accumulation could be possible. For AChE, no significant effects on activity rates could be detected for individuals exposed to fungicides in either sampling site. Thus, the possibility of neurotoxic effects caused by the studied fungicide formulations can probably be dismissed.

For the Touchdown® application, GST activity increased during day 2 after the application, in the sampling site of Longen. This would correspond to the effects measured for the fungicide applications. Since significant effects were only detected during days 2–4 for fungicide formulations, it is not that surprising that no significant effects were observed neither during day 1, 5 (Lörsch) or 7 (Fell). Again, an activity peak around day 3 after exposure with a subsequent normalization could be assumed (Knaebe et al., 2006). In contrast, GR activity was significantly higher in Fell during day 7 after the application took place. This could be an indicator towards oxidative stress caused by this glyphosate formulation, even one week after the initial exposure (an evident increase in activity can already be observed during day 5 after exposure in Lörsch, Fig. 2b). Thus, the effects of this pesticide on GR may be more lasting than the oxidative stress caused by fungicides.

Organophosphate pesticides like glyphosate formulations such as Roundup® have already been previously shown to have inhibiting effects on esterases, such as AChE and B-esterases in squamates (Amaral et al., 2012c; Sanchez et al., 1997). In the present study, a reduction of AChE activity rates can be observed during all days after exposure for all sampling sites (Fig. 2c, f, i). The inhibition rates reached really high levels (from 40% in Longen, to 89% in Fell), and are consistent with previous studies (Amaral et al., 2012c; Sanchez et al., 1997). While the results of the present study are not significant, this may be attributed to the low sample size available for this application due to bad weather conditions following the application. However, we can't make any decisive assertions.

For all of the reported effects, it is important to note that we do not know whether they are caused by the active ingredient/s, the adjuvants, or the whole pesticide formulation itself; in many cases, the adjuvants contribute more to adverse effects than the active ingredients (Cox and Surgan, 2006; Wagner et al., 2013). At the same time, we cannot conclude whether these effects may result in significant population level effects or not, as this would demand (1) a larger sample size, (2) the determination of toxicological endpoints and especially (3) long-term monitoring of the populations (sizes, reproductive success etc.) including potential co-factors apart from pesticide use, which can affect reptile populations.

However, our results are in accordance to previous studies regarding pesticide exposure to reptiles. In particular, a study by Amaral et al. (2012b) on a related wall lizard species, Bocage's wall lizard (*Podarcis bocagei*), exposed to different herbicides, provided evidence for increased GST and GR activity rates. Another study by Amaral et al. (2012c) on *P. bocagei* exposed to chlorpyrifos (i.e. aorganophosphorous insecticide), revealed a clear inhibition of carboxylesterases (CbE) and cholinesterases (ChE). Sanchez et al. (1997) observed similar results when studying Tenerife lizards (*Gallotia galloti*), exposed to the insecticide and acaricide parathion.

4.2. Saliva sampling via buccal swabbing as a minimal-invasive method for enzyme activity determination

Saliva sampling using buccal swabs was proposed and tested as a non-invasive method in human pesticide biomonitoring, using AChE as biomarker (Henn et al., 2006). Based on these experiences, we for the first time tested this method in biomonitoring of squamate reptiles exposed to pesticides. In addition, we applied it to GST and GR, for which no such studies are available.

The results imply that, in fact, saliva sampling via buccal swabbing could become a useful tool to determine pesticide exposure in reptiles, although further investigation is needed. While data regarding correlations between enzyme activity levels from saliva samples and internal organs (such as liver) or blood is needed in order to determine the efficiency of this method, it can be concluded, that buccal swabbing indeed seems adequate to at the very least detect exposure to pesticide formulations (i.e. that pesticides have indeed been taken up by the organism). At the same time, it is crucial to know when the exposure event took place, as a significant increase in GST activity was only detectable until day 4 after exposure, and GR only showed a narrow time margin in which significant differences were detectable (at day 3 after exposure, although still measurable at day 7 for the Touchdown® application). Salivary AChE has the potential become a very good indicator regarding the exposure to glyphosate-based herbicides (here Touchdown®).

While data concerning the relationship between salivary and liver (or blood) enzyme levels could not be measured, it is important to note that tissue samples (muscle) from lizard tail-loss during sampling did reveal a positive correlation relationship to salivary samples in GST and GR activities, but not for AChE. The latter may be explained by substantially higher concentrations of this enzyme in saliva when compared to muscle tissue and blood, as can be found in mammals (Ord and Thompson, 1950). Although we do not know if salivary AChE levels correlate with brain AChE levels, it can be argued that inhibition rates might be similar for both cases. Actually, inhibition rates from tail samples of lizards exposed to the Touchdown® herbicide were pretty similar to those measured in saliva (46% in tail samples vs 40% in saliva). In order to fully standardize this method, however, data on enzymatic activities from blood and internal organ samples is crucial. These samples could not be retrieved within our current study, but are needed in order to estimate how saliva relates to the “traditional” tools (Amaral et al., 2012b; Lajmanovich et al., 2008).

The success rate of enzymatic assays using buccal swabs was around 90%, indicating a good suitability of the method. We do not know, however, how this compares to standard techniques, as we do not possess this data for lizards.

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Finally, due to the fact that our findings are supported by results obtained in previous studies concerning the exposure of reptiles to pesticides, we conclude that the use of saliva from buccal swabs could in the future become a sensitive and minimal-invasive method for detecting pesticide exposure in reptiles. We see that this method has the potential to replace invasive methods, such as organ extraction or cardiac puncture that require euthanasia of individuals (Amaral et al., 2012b; Lajmanovich et al., 2008), although further research is needed. In this way, our results might stimulate this research field so that saliva sampling via buccal swabbing could even become a standard method for (squamate) reptiles risk assessment in pesticide admission procedures.

5. Conclusions

Reptiles are non-target organisms when considering effects of plant protection products. We could detect uptake of pesticides after their applications in common wall lizards living in vineyards, using previously established enzymatic biomarkers, but for the first time using a minimal-invasive sampling method, i.e. saliva sampled via buccal swabbing. Our results imply that exposed individuals suffer from oxidative stress caused by the applied formulations.

There is a need for reptiles to be integrated into risk assessments for pesticide admission procedures, in order to improve conservation practice. This requires that assessment methods are tested for the possibility to define standards. Saliva was shown to represent a promising medium to measure activity rates of the mentioned biomarkers. Buccal swabbing is minimally-invasive and has the potential to replace invasive methods in the future, such as organ extraction or cardiac puncture, also in other animal groups, such as amphibians.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2016.09.022.

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