Valentin Mingo

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The use of plant protection products and its impact on reptiles

Betreuender: Prof. Dr. Stefan Lötters

Berichterstattende: Prof. Dr. Stefan Lötters, Prof. Dr. Michael Veith, Dr. Carsten Brühl

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Abstract

Reptiles belong to a taxonomic group characterized by increasing worldwide population declines. However, it has not been until comparatively recent years that public interest in these taxa has increased, and conservation measures are starting to show results. While many factors contribute to these declines, environmental pollution, especially in form of pesticides, has seen a strong increase in the last few decades, and is nowadays considered a main driver for reptile diversity loss. In light of the above, and given that reptiles are extremely underrepresented in ecotoxicological studies regarding the effects of plant protection products, this thesis aims at studying the impacts of pesticide exposure in reptiles, by using the Common wall lizard (*Podarcis muralis*) as model species. In a first approach, I evaluated the risk of pesticide exposure for reptile species within the European Union, as a means to detect species with above average exposure probabilities and to detect especially sensitive reptile orders. While helpful to detect species at risk, a risk evaluation is only the first step towards addressing this problem. It is thus indispensable to identify effects of pesticide exposure in wildlife. For this, the use of enzymatic biomarkers has become a popular method to study sub-individual responses, and gain information regarding the mode of action of chemicals. However, current methodologies are very invasive. Thus, in a second step, I explored the use of buccal swabs as a minimally invasive method to detect changes in enzymatic biomarker activity in reptiles, as an indicator for pesticide uptake and effects at the sub-individual level. Finally, the last part of this thesis focuses on field data regarding pesticide exposure and its effects on reptile wildlife. Here, a method to determine pesticide residues in food items of the Common wall lizard was established, as a means to generate data for future dietary risk assessments. Subsequently, a field study was conducted with the aim to describe actual effects of pesticide exposure on reptile populations at different levels.

Thesis structure

This thesis addresses the question of how pesticide applications affect wild reptile populations, by evaluating exposure probability of reptiles to pesticides, establishing new methods to detect exposure scenarios using sub-individual biomarkers and quantifying contamination of food items, as well as studying the impacts of exposure at the sub-individual, individual and population level. According to these topics, this thesis is divided into three chapters. The first chapter addresses the risk of pesticide exposure for reptile species natively occurring within the European Union (EU). It consists of two GIS-based approaches through which the probability of exposure to plant protection products (PPP) was evaluated. The first part of Chapter I focuses on the exposure risk of protected European reptile species listed under Annex II of the habitats directive, within their special areas of conservation, and can be found in:

Wagner, N., **Mingo, V.**, Schulte, U., Lötters, S. 2015. Risk evaluation of pesticide use to protected European reptile species. Biological Conservation 191, 667-673.

In the second part, the focus shifts towards evaluating the risk of pesticide exposure of reptiles at the entire EU level, and encompasses an assessment of 102 natively occurring reptile species. The study further elucidates differences in exposure sensitivity between different reptile orders and suborders, as well as exposure probability for different reptile species inhabiting different pesticide admission zones ("Mutual Recognition Zones") within the EU, and was published in:

Mingo, V., Lötters, S., Wagner, N. 2016. Risk of pesticide exposure for reptile species in the European Union. Environmental Pollution 215, 164-169.

In Chapter II, the focus shifts from describing exposure risk and probability to a more practical way of detecting actual effects of pesticide exposure *in situ*. The chapter deals with the implementation of a new, minimal-invasive method, by making use of so called "buccal swabs", as a means to analyze enzymatic biomarkers of pesticide exposure. In a first field approach, the use of buccal swabs was tested by sampling Common wall lizards from different sampling sites that are regularly treated with pesticides (i.e. vineyards). The work can be found in:

Mingo, V., Lötters, S., Wagner, N. 2017. The use of buccal swabs as a minimal-invasive method for detecting effects of pesticide exposure on enzymatic activity in common wall lizards. Environmental Pollution 220, 53-62.

In a subsequent step, the proposed method was validated in a lower tier laboratory approach, by exposing wild caught Common wall lizards to different pesticide formulations, and analyzing enzymatic activities before and after an exposure event had taken place. Due to the presence of parallel control groups and the removal of potential unaccounted variables, the suitability of the method was fully validated. Furthermore, additional endpoints such as basking behavior, locomotor performance and feeding habits after different exposure scenarios were observed and linked to pesticide exposure. The study is currently *under review* in:

Mingo, V., Leeb, C., Fahl, A., Brühl, C., Lötters, S., Wagner, N. 2017. Validating buccal swabbing as a minimal-invasive method to detect pesticide exposure in reptiles. Environmental Pollution (*under review*).

Finally, Chapter III deals with the assessment of potential risks of pesticide exposure *in situ*. The first part of this chapter focuses on the quantification of pesticide residues in food items

of the Common wall lizard, at different time points after an application has occurred, in different sampling sites. It comprises the establishment of an analytical method to identify the risk of dietary pesticide exposure in reptiles, via residue analyses of multiple pesticides commonly applied in viniculture, for different prey items (i.e. insects, snails and spiders). The study can be found in:

Stöckelhuber, M., Müller C., Vetter, F., **Mingo, V**., Wagner, N., Lötters, S., Bracher, F. 2017. Determination of Pesticides Adsorbed on Arthropods and Gastropods by a Micro-QuEChERS Approach and GC–MS/MS. Chromatographia 80, 825-829.

The second part of this chapter aims to identify the actual effects pesticide exposure in wild reptiles, by monitoring effects of chronic exposure on sub-individual biomarkers, individual parameters and population level effects, using the Common wall lizard as model species. The results of the study were published in:

Mingo, V., Lötters, S., Wagner, N. 2017. The impact of land use intensity and associated pesticide applications on fitness and enzymatic activity in reptiles—A field study. Science of the Total Environment 590, 114-124.

This thesis was composed with the help of different people, which were involved in the study designs, data recompilation and analysis or composition of the manuscripts constituting the different chapters (Table 1).

Table 1: overview regarding the amount of work contributed by myself (%) to the studies comprising the different chapters of this thesis.

Co-Authors and people who were involved and/or collaborated in the composition of the different papers: Norman Wagner (NW), Stefan Lötters(SL), Christoph Leeb (CL), Carsten Brühl (CB), Ann-Katrin Fahl (AF), Markus Stöckelhuber (MS), Christoph Müller (CM), Franz Bracher (FB), Florian Vetter (FV).

Paper	Study design	Data recompilation	Data Analysis	Manuscript composition
"Risk evaluation of pesticide use to protected European reptile species"	10% NW, US	20% NW, US	0%	30% NW, US, SL
"Risk of pesticide exposure for reptile species in the European Union"	70% NW, SL	100%	100%	80% NW, SL
"The use of buccal swabs as a minimal-invasive method for detecting effects of pesticide exposure on enzymatic activity in common wall lizards"	80% NW	100%	100%	80% NW, SL
"Validation of a minimal-invasive method to detect pesticide exposure in reptiles – Linking pesticide exposure to enzymatic activity and behavior of Common wall lizards"	80% NW	100%	100%	80% NW, SL, CL, AF, CB
"Determination of Pesticides Adsorbed on Arthropods and Gastropods by a Micro-QuEChERS Approach and GC–MS/MS"	85% NW, CM	50% MS	0% MS, CM, FB, FV	20% MS, CM, FB, FV, NW, SL
"The impact of land use intensity and associated pesticide applications on fitness and enzymatic activity in reptiles—A field study"	70% NW, SL	100%	100%	80% NW, SL

During the course of my PhD-thesis, three additional peer-reviewed articles were composed, which revolved around herpetological questions concerning habitat suitability of cover crop plantations for reptile assemblages, potential effects of pesticide applications on future developments of reptile populations in Germany and effects of Bti applications on different developmental stages of the European common frog:

Carpio, A.J., López, J.C., **Mingo, V.**, Tortosa, F.S. 2017. Herbaceous cover enhances the squamate reptile community in woody crops. Journal for Nature Conservation 37, 31-38.

Mingo, V., Wagner, N. 2017. Der Einsatz von Pflanzenschutzmitteln in Deutschland: Auswirkungen auf Enzymaktivitäten und Populationsstruktur einheimischer Reptilienarten am Beispiel der Mauereidechse (*Podarcis muralis*). Zeitschrift für Feldherpetologie 24, 167-186.

Allgeier, S., Frombold, B., **Mingo, V.**, Brühl, C. 2017. European common frog *Rana temporaria* (Anura: Ranidae) larvae show subcellular responses under field-relevant Bacillus thuringiensis var. israelensis (Bti) exposure levels. Environmental Research (*under review).*

1 Summary

1.1 The global decline of reptiles

Global declines of biodiversity are an imminent threat for many taxa, including birds, mammals, amphibians and reptiles (Gibbons et al. 2000, Stokstad 2010, Cardinale et al. 2012). Regarding reptiles, 15-32% of worldwide diversity is estimated to be threatened (Böhm et al. 2013, http://www.reptile-database.org). At the European level alone, 20% of reptile diversity is classified as threatened according to the IUCN Red List of Threatened Species while, independently of these, 42% of all European reptile species display declining population trends (Cox and Temple 2009). However, reptiles remain the least studied taxa among vertebrate groups (Gibbons 1988, Bonnet et al. 2002, Baillie et al. 2004). This state becomes very clear when observing the conservation status of reptiles, which is unknown (Data Deficient) for the great majority of species and, especially, for squamates (http://www.iucnredlist.org, Sparling et al. 2010). According to current knowledge, reptiles are almost as strongly affected by global biodiversity loss as amphibians, a vertebrate group which has received a lot of attention in past years, mainly because of the critical state of many populations and the dramatic increase in declines (Houlahan et al. 2000, Collins and Storfer 2003, Stuart et al. 2004, Sparling et al. 2010, Hayes et al. 2010). While amphibians and reptiles have been traditionally studied under the field of herpetology, it has not been until recent years that interest in conservation of this group has started to increase (Baillie et al. 2004, Sparling et al. 2010). Regarding reptiles, the lack of social interest, or rather the social apathy concerning these taxa, has often been mentioned as a major factor negatively influencing conservation (Gibbons 1988, Todd et al. 2010), as they are often considered as a subject of personal derision. Thus, information regarding reptile declines is comparatively

lacking. However, many different factors influencing this loss in biodiversity have been identified:

- Habitat loss and degradation
- Anthropogenic environmental pollution
- Unsustainable removal
- Climate change
- Invasive species
- Disease and parasitism
- Cascading declines

It is important to note that these declines cannot be attributed to one single main cause, but are influenced by many different factors, which can act either independently or in combination. Nevertheless, these drivers share a common trait: human interaction (Gibbons et al. 2000, Todd et al. 2010).

1.2 Causes for reptile declines

Habitat loss and degradation is considered the single most important factor affecting reptile declines (Mittermeier et al. 1992, Gardner and Oberdörster 2006). By negatively influencing habitat suitability, it limits the ability of reptiles to match their ecological needs, by eliminating foraging and refuge areas, or limiting food availability and basking spots (Sparling et al. 2010). Similarly, habitat fragmentation causes discontinuities in the preferred environment of a certain species, by isolating patches of suitable habitat and/or limiting access for important parts of the populations (Dodd 1991, Kjoss and Litvaitis 2001, Driscoll 2004). Furthermore, fragmentation can repress the demography of remaining populations by geographically segregating them (Hokit and Branch 2003). However, habitat loss and

degradation only constitute a part of the problem. Introduction of invasive species due to anthropogenic expansion is considered a great threat towards global biodiversity and has, in fact, already resulted in severe ecological damage (Pimentel et al. 2000, Park 2004). Invasive species may influence biodiversity by direct predation of native species (Henderson 2004), through habitat modification (Brooks and Pyke 2001, Valentine 2006) or competition (Cadi and Joly 2003). At the same time, it can be an important vector for additional repressing factors, such as disease and parasitism. Normally, species are more adapted to pathogens within their natural distribution range, thus limiting potential devastating effects on entire populations (Gibbons et al. 2000, Collins and Storfer 2003). However this may not be the case for "unknown" pathogens, to which native species are not adapted to. Disease outbreaks initiating declines have already been documented, and are of particular concern for turtles. A prime example is the upper respiratory tract disease infection in several different tortoise species, which was probably introduced into natural populations by the release of infected captive individuals (Dodd and Seigel 1991, Seigel et al. 2003).

Climate change is yet another important factor regarding conservation of biodiversity, not only for reptiles, but all organisms, and has often been discussed as one of the major challenges of the Anthropocene (Steffen et al. 2011). Indeed, climate change also has the potential to severely affect reptile populations, by directly interfering with their ability to thermoregulate, thus influencing growth rate and age to reach sexual maturity (Frazer et al. 1993). Indirect impacts, such as the influence in sexual determination may further alter population structures (Janzen 1994), cause changes in habitat suitability (causing shifts in reptile distributions at a large scale), or lead to extinction events if a relocation cannot be achieved (Araújo et al. 2006). Although climate change per se is a natural process that has accompanied reptiles for the past 65 million years (Zachos et al. 2001), the speed at which

recent (anthropogenic) climate warming is occurring (IPCC 2007) has to be considered a great threat for reptiles, as adaptation will not be possible at the current pace.

Reptiles are also susceptible towards declines in other taxa, commonly known as so called cascading declines (Gardner and Oberdörster 2006). This is because the loss of important species within an ecosystem can severely impact previously unaffected species. For example, the extreme decline of amphibians may be responsible for some of the ongoing reptile declines, as they are being robbed of their prey (Matthews et al. 2002, Whiles et al. 2006, Toledo et al. 2007). Again, these amphibian declines may, in many cases, be caused by anthropogenic factors themselves (Stuart et al. 2004).

Finally, environmental contamination has been gaining much prominence, especially in the last few decades, and represents a major factor that can be almost exclusively be linked to human action. It is currently considered one of the most imminent threats for reptile diversity, especially in industrialized countries (Gibbons et al. 2000, Sparling et al. 2010). Regarding environmental contaminants, great emphasis is given to pesticides due to the sheer and diverse amounts of formulations and active substances continuously applied all over the globe (Pimentel 1995, Alavanja 2009, Sparling et al. 2010). Pesticides are substances with the primary function to control target pests or weeds, by incapacitating and/or killing them (Gilden et al. 2010). Yet, the existence of multiple reports regarding detrimental effects of pesticides on non-target wildlife is of great concern (Iyaniwura 1991, Pimentel 1995, Sparling et al. 2010, Johnson and Gnanadhas 2016). Undeniably, numerous cases of detrimental effects of pesticide exposure have already been described for several reptile species, both *in situ* and *ex situ* (Willemsen and Hailey 2001, Weir et al. 2010, Amaral et al. 2012a,b, Latorre et al. 2013, Weir et al. 2014, Cardone 2015, Douros et al. 2015, Weir et al. 2015, Carpenter et al. 2016). Nevertheless, these taxa have been largely neglected in ecotoxicological studies

regarding the effects of pesticide exposure up to this day (Hopkins 2000, Campbell and Campbell 2002). Environmental contamination thus poses a major threat for reptile biodiversity. However, even now, it is still a largely understudied research area which has the potential to greatly affect reptile biodiversity.

1.3 Reptiles in agricultural landscapes

As already explained, habitat loss and degradation are considered the most important drivers for global reptile declines (Dodd 1990, Mittermeier et al. 1992, Kjoss and Litvaitis 2001, Driscoll 2004, Gardner and Oberdörster 2006). Within industrialized countries, habitat loss has historically been greatly conditioned by the expansion of agriculture, housing and infrastructural development (Todd et al. 2010). In Germany, almost 50% of the country's area is dedicated to agriculture (http://www.destatis.de) whereas at the EU scale, about 40% of the entire land area is dedicated to agronomy (http://www.ec.europa.eu) and greatly overlaps with the distribution of many reptile species. However, many reptiles have managed to persist and adapt to these new, altered habitats. In fact, various species do occur within agricultural landscapes (Fryday and Thompson 2009, Biaggini and Corti 2015). A recent study has shown that for at least 27% of occurring species (40 out of 141), direct evidence is available indicating their regular presence within agricultural areas (Mingo et al. 2016). At the same time, these populations are, in the more recent time, confronted with the ever increasing use of anthropogenic environmental pollutants in agriculture, namely pesticides and other agrochemicals (Gibbons et al. 2000, Gibbs et al. 2009, Sparling et al. 2010). As a matter of fact, Germany occupies 4th place in the EU ranking regarding application quantity of plant protection products (PPP), with a total output of roughly 25.000 tons of active substance (a.s.) per year (Eurostat 2007). At the EU level, the total amount of PPP applied per year amounts to 219.662 tons of a.s.. Yet, the majority of these applications are concentrated in southern

countries of the Union, namely France, Italy and Spain. These countries occupy the rankings 1st to 3rd, respectively, and make up to 56% of the entire pesticide output (Eurostat 2007). Simultaneously, the greatest abundance and diversity of reptiles can be found in southern countries of Europe (Cox and Temple 2009).

For reptiles, one of the main aspects concerning exposure towards pesticides is their life-history, as they display a rather low dispersal capability, with small home ranges and in multiple cases, territoriality (Simon 1975, Southwood and Avens 2010). This implies that potential exposure patterns for sedentary species can be defined rather easily, with populations inhabiting agricultural landscapes suffering repeated, almost chronic exposure events, while those inhabiting non-exposed areas will have low chances of coming into contact with agrochemicals (EFSA 2017). Nonetheless, there have also been reported cases where snakes and turtles have been attracted to crop fields for nesting, due to their preference for loose soils during egg laying and incubation, although generally not occurring within agricultural landscapes (Kaufmann 1992, Wisler et al. 2008).

Finally, although many reptile species within the EU benefit from protection by special conservation programs, such as the Habitats Directive (Council Directive 92/43/EEC), no direct consideration is given regarding possible effects of pesticide exposure. Thus, in an aim to discern the probability of pesticide exposure and to evaluate the potential impacts of pesticide applications on reptile species, Chapter I presents two studies in which the general occurrence of reptiles within agricultural areas was evaluated, and their risk of pesticide exposure was assessed:

1.3.1 Risk evaluation of pesticide use to protected European reptile species

Many European reptiles are listed under Annex II of the Habitats Directive (Council Directive 92/43/EEC). This Annex lists species of community interest and whose conservation requires the designation of special areas of conservation (SACs). SACs are described within the Natura 2000 network, which has the conservation of Europe's natural heritage (i.e. threatened species and habitats) as its main goal. Within these SACs, member states take the responsibility of ensuring a favorable conservation status of species and habitats, including regular monitoring and management plans (http://ec.europa.eu). However, agricultural land use does not stop at SAC borders and land use within them is possible under certain conditions (Council Directive 92/43/EEC). Concerning reptiles, 21 species and 3 subspecies are recognized under Annex II of the Habitats Directive, seven of which are so called priority species, which require enhanced protection. The aforementioned conservation requirements for the protection of reptiles, however, do not take the effects of pesticide exposure caused by agricultural land use directly into account. In an aim to clarify whether current land use practice with regular pesticide applications is likely to affect Annex II reptiles within their SACs, a risk evaluation was conducted at the European level. Pesticide exposure risk depends on multiple factors such as life history and biology of species, but also on SACs of the different member states. Thus, the amount of agriculture with regular pesticide applications within every national SAC, for each reptile species listed under Annex II, was evaluated. Coupled with a species risk index and pesticide risk factor, derived from the actual occurrence within agricultural landscapes, as well as the biology and life history of a species, the following questions were addressed:

"How high is the individual risk of pesticide exposure for each species, and are there observable differences between member states?"

Out of all 21 reptile species listed under Annex II of the habitats directive, nearly half are considered threatened in their global distribution by the IUCN Red List of Threatened Species, while 7 are under the status of 'near threatened', and only 4 are listed under the category 'least concern'. Thus, additionally, we further addressed the following question:

"Are there differences in risk between conservation status and priority species?"

Of the 21 evaluated reptile species, 10 displayed above average exposure risks, within their SACs. Among these, 4 are listed as at least Vulnerable by the IUCN Red List of Threatened Species. However, the majority of Annex II species are listed as Near Threatened. At the same time, a great variability between proportions of agricultural land use were observed between SACs of different member states, strongly arguing for site- and species-specific evaluations in order to prevent regional biodiversity loss. According to these findings, we were able to confirm that even strictly protected species under Annex II of the habitats directive, for which special conservation programs have been implemented, are not 'safe' from pesticide exposure. Furthermore, SAC variability between member states makes general comparability troublesome, thus requiring specific assessments for each one.

The results of this study were published in the peer reviewed Journal "Biological Conservation" in the year 2015.

1.3.2 Risk of pesticide exposure for reptile species in the European Union

Concerning reptile species listed under Annex II of the habitats directive, we now know of the individual pesticide exposure risk. However, protected species are not the only ones threatened by environmental contamination, and are surely not the only ones that may suffer of detrimental effects on individual and/or population level. Thus, the logical next step is to

assess the risk of pesticide exposure for as many reptile species as possible. Here, we asked ourselves:

"C*onsidering all reptile species occurring within the EU, how many of them display an above average pesticide exposure risk?"*

To answer this question, a similar approach to that described in our previous study (Wagner et al. 2015) was employed, using a spatial risk evaluation approach, however, with some changes. A total of 141 reptile species are native within the EU (Cox and Temple 2009), out of which for 102, sufficient data was available to evaluate the exposure risk. In contrast to Wagner et al. (2015), presence within agricultural landscapes was assessed via literature research. For biological factors affecting exposure, snout to vent length (SVL) and body mass (BM) were employed as surrogates of physiology, since these factors have high influence on dermal uptake and oral exposure to pesticides. As for species' life history, the amount of clutches laid per year, as well as the number of eggs/descendants per clutch were used to assess population susceptibility towards pesticide exposure. Using all these data, an Exposure Risk Index (ERI) and Exposure Risk Factor (ERF) were calculated. The ERI specifically defines the susceptibility of a species towards pesticide exposure, while the ERF reflects the potential pesticide exposure risk according to habitat exposure, physiology and life-history, as well as proportion of agricultural area within its European distribution. Different reptile orders and suborders are characterized by rather important differences in ecology and physiology, as well as home ranges, thus raising the following question:

"Are there differences in exposure sensitivity between reptile orders and suborders?"

Finally, the EU is subdivided into three zones (South, Central, North) regarding admission of new plant protection products, the so called ‘Mutual Recognition Zones’ (EC Regulation No. 1107/2009). Hence, a final question was addressed:

“Are there differences between species’ occurrences within agricultural habitats amongst ‘Mutual Recognition Zones’?”

The results of the study revealed that at least one third of all reptile species occurring within the EU show an increased pesticide exposure risk. However, only two of them are considered under the category ‘Threatened’ (Vulnerable) by the IUCN Red List of Threatened Species. Regarding exposure sensitivity, the results imply that the reptile suborder lacertilia displays the greatest sensibility, which can be explained by their life-history and physiology. Concerning the ‘Mutual Recognition Zones’, the highest amount of reptile species occurring within agricultural areas could be observed within the southern zone. This may be explained by the higher amount of reptile species occurring within Mediterranean countries and the subsequent higher number of potential species inhabiting agricultural land. Given that diversity is greatest in southern countries of the EU, and the amount of pesticide applications within these is greatest in all of the Union, integration of reptiles into pesticides risk assessments seems indispensable in order to improve conservation.

The study was published in the year 2016 in the peer reviewed journal “Environmental Pollution”.

1.4 The current status of reptiles in pesticide ecotoxicology

Reptiles have been long neglected in ecotoxicological studies regarding the effects of pesticide exposure (Hopkins 2000, Campbell and Campbell 2002, Sparling et al. 2010). Until

the year 2000, reptiles only made up 1% of all ecotoxicological studies concerning this topic (Hopkins 2000). Simultaneously, these studies mainly focused on the reptile orders of crocodylia and testudines, largely overlooking the order of squamata (Campbell and Campbell 2002, Sparling et al. 2010). Yet, almost 95% of all 10.450 currently described reptile species correspond to this order, with almost 60% belonging to the suborder lacertilia (http://www.reptile-database.org). As our own risk evaluation has shown (Chapter I), squamate reptiles, especially those belonging to the suborder lacertilia, display the overall highest susceptibility towards pesticide exposure (Mingo et al. 2016). While interest in ecotoxicological effects of pesticide applications in this group of reptiles has increased ever since (Sanchez-Hernandez and Sanchez 2002, DuRant et al. 2007, Weir et al. 2010, Amaral et al. 2012a,b, Bicho et al. 2013, Weir et al. 2014, Weir et al. 2015, Cardone 2015, Carpenter et al. 2016, Schaumburg et al. 2016, Chang et al. 2017, Yanes-Marichal et al. 2017), knowledge regarding effects of exposure in lizards still remains rather uncharted, especially when compared to birds, mammals and fish, but also amphibians.

The use of enzymatic biomarkers of pesticide exposure has become a widely used approach to detect potential effects of pesticide uptake in individuals, both *in situ* and under laboratory conditions. These biomarkers have the advantage of generating information regarding chemical uptake and toxicant metabolism, as well as disclosing the impacts of pesticide exposure at the sub-individual (cellular) level. It has thus become a popular technique in ecotoxicology, in most cases coupled with additional, non-enzymatic endpoints. However, one of the main problems with which researchers are confronted whilst studying these markers is the invasiveness of sampling procedures the current methodology requires, such as organ or blood extraction (Amaral et al. 2012b, Bicho et al. 2013, Lajmanovich et al 2011), therefore potentially severely impairing individuals of populations we are trying to protect.

Especially concerning protected and endangered species, this poses a great dilemma, since it is wishful to know whether exposure has detrimental effects on individuals, but assessing sufficient individuals to reach reasonable conclusions would, in many cases, imply severely impairing the studied populations. At the same time, EU legislation regarding animals used for scientific purposes is strict (European Parliament and Council 2010). Conducting field studies to assess effects of pesticide exposure are therefore limited to few individuals in order to minimize animal suffering and to not impair conservation, especially when dealing with protected species (Council Directive 92/43/EEC).

Hence, the need to improve current methodologies and allow for natural reptile populations to be studied without having to worry about animal welfare and conservation aspects becomes rather clear. In an attempt to reduce invasiveness of current methodologies, Chapter II deals with the testing and validation of a minimally-invasive method to detect effects of pesticide exposure on biomarker activity in reptiles, by making use of buccal swabs as an alternative sampling method to blood and organ tissue samples:

1.4.1 The use of buccal swabs as a minimally-invasive method to detect effects of pesticide exposure in the Common wall lizard

The use of saliva as a means to detect effects of pesticide exposure had been previously proposed by Henn et al. (2006) in human pesticide biomonitoring. Similarly, Schulte et al. (2011) proposed the use of buccal swabs as a reliable method for DNA sampling and analysis. Based on these observations, we asked ourselves if this approach is suitable to detect enzymatic biomarkers of pesticide exposure in reptiles, by using the Common wall lizard (*Podarcis muralis*) as model species. To this end, a field study was carried out during the year 2015. Three sampling sites (vineyards with regular pesticide applications) were surveyed, and

a total of 245 individuals were analyzed by using buccal swabs. According to the goal of the study, the following question was addressed:

"*Do saliva samples / buccal swabs represent a suitable sampling method to detect enzymatic markers of pesticide exposure in reptiles?"*

Current methodology suggested that enzymatic biomarkers of pesticide exposure may only be reliably detected in blood or organ tissue samples. Previously described enzymatic assays were subsequently adjusted to this new sample type in order to conduct analyses. However, being able to detect enzymatic activity in saliva samples does not necessarily equal being able to detect effects of pesticide exposure. Subsequently, the following question arised:

"If enzymatic activity can indeed be detected by using buccal swabs, can changes in biomarker activity be observed following an exposure event?"

In order to answer this question, lizards were sampled from each study site within one week after an exposure event had taken place, during multiple applications. Changes in enzymatic activity were then observed during the days after an exposure event had occurred and compared with reference values gained from non-exposed individuals of each respective population. According to these changes in activity, a further question was addressed:

Can detrimental effects be observed at the sub-individual / cellular level?"

As tissue samples from a few individuals (autotomized tails during sampling) were available, we further asked ourselves the following question:

"Do tissue and saliva samples of the same individuals correlate regarding enzymatic biomarker activity?"

We were able to demonstrate that reptiles, being non-target organisms of pesticide applications, suffer form pesticide uptake within their natural habitats by using previously established enzymatic biomarkers, but for the first time by employing a minimally-invasive sampling method, i.e. buccal swabs. Results strongly matched to those obtained in independent laboratory studies concerning reptiles and other taxa. Changes in enzymatic activity could be linked to different pesticide application events. We were able to observe detoxification processes and increasing oxidative stress after exposure to pesticides, as well as indications towards potential neurotoxicity stemming from a herbicide application. At the same time, data gained from saliva samples matched that of tissue samples retrieved from autotomized tails of the same individuals, altogether indicating a good suitability of the method to detect pesticide exposure in reptiles.

The study was published in the peer reviewed journal "Environmental Pollution" during the year 2017.

1.4.2 Validating buccal swabbing as a minimal-invasive method to detect pesticide exposure in reptiles.

In a first step, using saliva samples/buccal swabs to detect potential effects of pesticide exposure was tested and carried out under field conditions. This was necessary as a means to establish whether the methodology is actually suitable to give information regarding pesticide exposure and effects *in situ*. While field studies have the advantage of actually reflecting natural conditions, standardization is very difficult, as a multitude of biotic and abiotic parameters can often not be taken into account, thus potentially influencing the observed endpoints (Mann et al. 2009, van Leeuwen and Vermeire 2010, Sparling et al. 2010). Although our previous study generated very good and plausible results, it is indispensable to

fully validate the methodology by excluding any external, unaccounted factors. Hence, a lower tier laboratory experiment in which Common wall lizards were exposed to environmentally relevant pesticide concentrations was conducted. By excluding any non-standardized parameters, changes in enzymatic activity after exposure to different pesticide formulations was directly linked to pesticide exposure itself. In an attempt to not only test whether buccal swabbing is a reliable technique to detect pesticide exposure, but also if it reflects both main exposure pathways (the oral and dermal pathways), lizards were divided into three treatment groups (control, dermal and oral exposure). Accordingly, the main questions to be answered within this study were:

"Are changes in enzymatic activity measured by using buccal swabs linked to pesticide exposure?"

"Is this methodology suitable to detect effects of oral and dermal pesticide uptake?"

Additionally to this method validation, further endpoints were studied to discern whether regularly applied pesticides, at conventional field doses, have a significant impact on behavior and locomotor performance of individuals – effects which have been previously observed under field and laboratory conditions. Accordingly, we asked ourselves the following questions:

"Do pesticide applications have an impact on mobility and locomotor performance of exposed Common wall lizards?"

"Does exposure to pesticides influence thermoregulation and food consumption of exposed individuals?"

We were able to validate the use of buccal swabs as a reliable method to detect pesticide exposure using different enzymatic biomarkers in reptiles. Given that any additional stressors which may have interfered in previous field studies were eliminated in this laboratory approach, it can be concluded that results gained in our previous study were indeed caused by pesticide exposure. At the same time, similarity between observed effects and field data is remarkable. Buccal swabbing thus represents a good alternative to explore exposure and potential effects of pesticide formulations in reptiles. Due to its minimal-invasiveness, the method allows to test a much higher amount of individuals, opening new possibilities for study designs. Concerning the behavioural endpoints, a decrease in locomotor performance was observed for individuals exposed to a fungicide mix, although the exact mode of action remains unclear. At the same time, increased basking activity was observed in exposed individuals, whereas no avoidance of contaminated food items was detected. Overall, these effects could lead to an increased mortality in exposed populations under natural conditions.

The manuscript has been submitted to the peer reviewed journal “Environmental Pollution” and is currently being reviewed.

1.5 Effects of pesticide exposure and exposure pathways in reptiles

Although reptiles are largely understudied concerning effects of pesticides, there have been multiple reports of potentially lethal and sub-lethal implications in exposed individuals, even at environmentally relevant concentrations (Weir et al. 2015). Here, both field and laboratory studies have shown a plethora of adverse effects. Regarding squamate reptile species, research has only started to increase recently. However, numerous effects have already been been observed, such as impairments in fertility in Italian wall lizards (*Podarcis sicula*) (Cardone 2015). Similarly, Amaral et al. (2012a,b) detected a general loss of body condition, disturbed

sex ratios and oxidative stress in Bocage's wall lizards (*P. bocagei)* inhabiting agricultural habitats regularly treated with pesticides. At the same time, Bicho et al. (2013) observed changes in thyroid activity within *P. bocagei* exposed to different pesticide formulations. Thyroid hormones play a major role in lizard growth and are directly involved in many physiological processes. Alterations in activity can thus have severe consequences on growth and development of individuals. Schaumburg et al. (2016) witnessed genetoxicity in Tegu lizard embryos (*Salvator merianae*) exposed to different glyphosate-based herbicide formulations, while Hopkins and Winne (2006) perceived impairments in swimming performance of natricine snakes exposed to the insecticide carbaryl. Carpenter et al. (2016) on the other hand reported "fever responses" in skinks (*Oligosoma polychroma*) exposed to glyphosate-based herbicide formulations. Regarding non-squamate species, Latorre et al. (2013) observed impairments in the immune system of young Caimans (*Caiman latirostris*) after exposure to Roundup®, while Beldomenico et al. (2007) further described an increased loss in egg weight and hatchling weight after *in ovum* exposure to atrazine and endosulfan within the same species. Similarly, Poletta et al. (2011) noticed clear signs of genotoxicity and alterations in metabolism, as well as growth delay in caimans exposed to a glyphosate based formulation, and a formulation mix containing glyphosate, endosulfan and cypermethrin. Concerning alligators, Guillette et al. (1994) reported significant alterations in embryonic sexual development in *Alligator mississipiensis* inhabiting Lake Apopka, a heavily pesticide contaminated water body containing DDT and its metabolites DDD and DDE, strongly depressing subsequent reproductive success of individuals. The authors concluded that these chemicals can work at various biological levels, from outright mortality of eggs and adults, to sub-lethal effects such as changes in gonadal status. Lind et al. (2004) found abnormalities in bone composition of female alligators of the same lake (i.e. increased bone mass), altering their morphology and physiology. Further studies demonstrated that parental exposure to

organochlorine pesticides is linked to decreased clutch viability in *A. mississipiensis*, raising great concerns for endangered crocodilian species inhabiting pesticide contaminated habitats (Rauschenberger et al. 2007). As for the order of testudines, Willingham and Crews (1999) reported sex reversal effects in eggs of the Red-eared slider turtle (*Chrysemis nelsoni*) incubated at male producing temperature, but exposed to different pesticides. Willingham (2001) further observed severe impairments in growth rate within the same species after exposure to chlordane, trans-non-alachlor and p,p'-DDE, at low doses. These findnigs indicate that these compounds seem to have a significant impact regarding endocrine disruption that extends beyond sex determination and development. Willemsen and Hailey (2001) perceived clear symptoms of poisoning and increased mortality in the Herman's tortoise (*Testudo hermanni)* after exposure to the herbicides 2,4-D and 2,4,5-T, while Tangredi and Evans (1997) witnessed immunosuppressive effects of low level exposure to organochlorines in Eastern box turtles (*Terrapene carolina*).

Many routes of exposure have been discussed for reptiles. For instance, chemical uptake can already begin shortly after oviposition (Sparling et al. 2010). Studies have demonstrated that some reptile species are attracted to farmland for nesting, as soil is more loose and easier to burrow, thus increasing exposure risk through egg shell absorption from the egg's surroundings (Wisler et al. 2008, Gardner and Oberdörster 2006). However, exposure to organic and inorganic contaminants may even take place before oviposition, by direct maternal transfer during vitellogenesis, as reported by Pagano et al. (1999) and Nagle et al. (2001). Here, large amounts of lipoproteins which are essential for supplying nutrients are synthetized during pregnancy, and may act as vectors for contaminant transport from mother to offspring (Hopkins 2006). Dermal uptake of pesticides on the other hand has long been considered a negligible exposure route for reptiles, since the general opinion was that reptile

skin is relatively impermeable due to the high amounts of keratin within the skin (Snodgrass et al. 2008, Sparling et al. 2010). However, this is far from the truth. Reptilian skin varies tremendously in permeability, primarily as a function of cutaneous lipid layers (Hopkins 2006), with species that have reduced lipid layers being more prone towards uptake of polar compounds, whereas species with thicker lipid layers may be more prone to absorption of lipophilic substances. Indeed, studies have shown that skin permeability in reptiles is almost completely dependent on lipid levels, and not keratin (Roberts and Lillywhite 1980, Tu et al. 2002, Toni and Alibardi 2007, Weir et al. 2010). As reptile skin is normally characterized by high lipid content, uptake of hydrophilic contaminants will mostly be prevented, whereas lipophilic ones will be absorbed (Pough et al. 2016). Weir et al. (2014) recently showed that differences in tissue residue concentrations of different model chemicals didn't drastically differ between oral and dermal exposure routes. The authors came to the conclusion that dermal exposure is probably an especially important uptake pathway for reptiles, as their poikilothermic physiology equals to lower energetic demands, and thus a lower dietary exposure as opposed to endotherm vertebrates such as birds and mammals. Aside from dermal exposure, oral uptake of pesticides constitutes the second "main" exposure pathway. The most important uptake mode regarding oral exposure is the ingestion of contaminated prey items (Hopkins 2006, Gardner and Oberdörster 2006, Sparling et al. 2010). However, generalization regarding uptake intensity is difficult. Reptiles occupy differing trophic levels and display diverging feeding ecologies. Biomagnification probably poses a high risk to reptiles in high trophic levels (predators), as has been reported in previous studies (Meyers-Schöne et al. 1994, Campbell 2003, Sparling et al. 2010). Scavenging reptiles which prey from carrion on the other hand, may suffer from increased exposure levels due to ingestion of contaminant levels which were lethal to prey (Hopkins 2006). Similarly, herbivorous reptiles are not only susceptible towards contaminants accumulated in plants, but also for example to pesticides

that adhere on plant surfaces. At the same time, trophic transfer in herbivores is largely understudied compared to carnivores (Hopkins 2006). Finally, soil ingestion has been discussed as a potential oral uptake pathway regarding environmental contaminants. Ingestion of substrate has been widely observed in reptiles, although the purpose of this behavior is yet unclear (for instance obtaining micronutrients, macerating food or maintaining the intestinal microflora) (Sokol 1971, Sylber 1988, Beyer 1994).

Out of all discussed scenarios, the general consensus is that the dermal and oral routes of exposure play the most significant role in reptile ecotoxicology, due to the comparatively high amount of pesticides that can be absorbed through these pathways (Hopkins et al. 2006, Sparling et al. 2010, Weir et al. 2010).

Knowing that reptiles may suffer from pesticide uptake via different exposure routes, and that a great variety of detrimental effects of pesticide exposure have been documented, Chapter III focuses on identifying the effects pesticide exposure has on a widely distributed reptile species that regularly occurs within agricultural habitats (*P. muralis*). Pesticide residues were quantified in food items of the Common wall lizard as a means to identify the dietary pesticide exposure risk, by establishing an analytical method to detect pesticide residues in insects, snails and spiders. The second part of this chapter deals with the detection of effects of pesticide exposure at the sub-individual, individual and population level in Common wall lizards. Here, studied individuals were exposed to pesticides via direct overspray and ingestion of contaminated food items within the scope of regular vineyard treatments.

1.5.1 Determination of Pesticides Adsorbed on Arthropods and Gastropods by a Micro‑QuEChERS Approach and GC–MS/MS

In Chapter II, we already discussed the importance of exposure pathways whilst assessing the effects of pesticide exposure on enzymatic biomarkers, locomotion and behavior, by dermally and orally exposing common wall lizards to different pesticide formulations (Mingo et al. *under review*). However, this data does not allow to quantify residue levels in individuals or food items. Quantification of residue levels in wild caught wall lizards would require euthanasia of individuals in order to conduct analysis. Due to the restrictiveness of EU legislation regarding animals used for scientific purposes (European Parliament and Council 2010), as well as protection status of reptile species listed under Annex IV of the habitats directive (Council Directive 92/43/EEC), direct analysis of residues in wild living reptiles is not feasible. Regarding pesticide residues in Common wall lizards, during the year 2015, we found four dead animals within a sampling site, shortly after a fungicide application had taken place. In accordance with the nature conservation agency SGD Nord, residue analyses were conducted at the Department of Pharmacy of the Ludwig-Maximilians University in München. Individuals were screened for active substances of regularly applied pesticide formulations in vineyards. According to the analyses, the active substance Quinoxyfen was detected in internal organs (liver, muscle and fat tissue) of 2 out of 4 individuals, whilst the active ingredient Difenoconazole was measured in liver of the remaining two wall lizards (Mingo et al. *unpublished data*). However, aside from these isolated cases where individuals were found shortly after being deceased, extraction of individuals from natural environments would be deemed a strong inference with populations. Thus, we steered away from analyzing pesticide residues in reptiles themselves, and focused on their prey items, being a major route for pesticide uptake (Hopkins 2006, Sparling et al. 2010). Although contaminated food items

are recognized as a major pesticide exposure pathway for not only reptiles, but also birds and mammals (EFSA 2009), only very few methods dealing with pesticide analysis of arthropods and gastropods, which belong to the main prey items of the Common wall lizard (Schulte et al. 2008), have been described in the literature. In order to evaluate the exposure risk of *Podarcis muralis*, the residue unit dose of its prey animals was determined at different times after exposure to pesticides. To this end, the active substances Cyflufenamide, Difenoconazole, Dimethomorph, Fluopicolide, Fluopyram, Metrafenone, Myclobutanil, Quinoxyfen, and Tebuconazole were used for method calibration, as these active substances are found in pesticide formulations that are commonly used in viniculture. Sampling of prey items took place throughout the entire activity period of *P. muralis* during the year 2016, in accordance with pesticide applications of surveyed vineyards, which were kindly provided by Daniel Regnery. Samples were then analyzed at the Department of Pharmacy at the Ludwig-Maximilians University in München.

The scope of the study encompassed two main goals as a means to assess the oral pesticide exposure risk for *P. muralis* via food items:

"To develop a simple, efficient and rapid method to detect pesticide residues in prey items of the common wall lizard"

"To determine whether pesticide residues can be measured in food items of the Common wall lizard"

Accordingly, the following question could be addressed:

"Are prey items of the Common wall lizard subjected to pesticide exposure via spray applications, and thus pose a risk when consumed?"

The goal of this study was to develop a miniaturized analytical method (QuEChERS technique) with which pesticide residues can be determined even in low amounts of reptile prey items. The method was fully validated for 9 widely used fungicides, two of which could be detected in prey items of *P. muralis.* Given the scarcity of described methods to analyze these compounds in arthropods and gastropods, the method is a first, important step towards standardization of dietary risk assessments for reptiles in intensively farmed environments.

The study was published in the peer reviewed journal "Chromatographia" during the year 2017.

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

According to the data gathered from all previous studies, we know that reptiles, and in this case, Common wall lizards, do not only exhibit a high risk of pesticide exposure, but indeed suffer from pesticide uptake, as described in Chapters I, II and III. Additionally, the reptile suborder lacertilia was shown to be the most sensitive one regarding pesticide exposure (Mingo et al. 2016). Yet, one question remains to be answered:

"Does pesticide exposure actually have a relevant effect at the individual, and most importantly, at the population level regarding the Common wall lizard?"

In an attempt to recompile as much data as possible on the effects of long term pesticide exposure on population parameters and individual, as well as sub-individual effects in reptiles, we studied four populations of *Podarcis muralis*, which were selected according to an agricultural gradient: from a non-exposed reference site, to a maximally exposed population surrounded by high agricultural intensity.

Effects of land use intensity and associated pesticide applications were used as indicators for individual, but also population specific effects. Our goal was to establish a clear link between intensity of pesticide applications and effects on population structure. All of the exposed sampling sites (vineyards) were characterized by the fact that they have been used for viniculture for more than 30 years, and pesticide applications take place during a major part of the year, from April until September, in order to combat pests (mainly fungi and weeds).

In order to study sub-individual effects of pesticide exposure, individuals were sampled according to the methodology presented in Chapter II. Concerning individual effects, biometric data was retrieved for each caught individual and compared between different sampling sites. Subsequently, these data were used to assess impacts of pesticide exposure at the population level. Body condition indices were calculated for individuals of each population, as a surrogate for fitness. Additionally, age structure and gender distribution was assessed and compared between all studied populations. Lizard sampling took place during the entire activity period of *P. muralis* during the year 2016. Furthermore, data regarding SVL, BM and gender retrieved from within the same populations during the year 2015 (Mingo et al. 2017, Chapter II) were used to assess differences in population structure during the years 2015 and 2016.

Hence, in this study, the following questions were addressed:

"*Does pesticide exposure induce ecotoxicologically relevant effects at the sub-individual level in the Common wall lizard?"*

"Are body condition and fitness of reptiles influenced by land use intensity and associated pesticide applications?"

"Does long term pesticide exposure affect population structure of the Common wall lizard?"

We were able to verify that exposure to pesticides induces oxidative stress in exposed individuals, within their natural habitat. This oxidative stress has in the past been linked to potentially severe implications for individuals. However, neurotoxicity could not be observed. A signifcant decrease in body condition was identified along the agricultural gradient, with increasing land use intensity (and presumably pesticide exposure). Similarly, age classess followed a comparable trend, with higher age classess in less exposed habitats. This can be seen as an indicator for increased mortality in areas with higher exposure intensity. While the Common wall lizard is most probably able to cope with these effects thanks to its ecology and life history, the same may not be be applied to other reptile species, such as e.g. the Western green lizard (*Lacerta bilineata*). This species only persists in small and remote populations in Germany, while also occupying vineyards as its main habitat. However, it is characterized by much lower population densities. An extrapolation of effects from the Common wall lizard to the Western green lizard would therefore most probably lead to much more severe effects at the population level. Consequently, reptiles urgently need to be integrated in pesticide risk assessments in order to properly assess the impact plant protection products have on these taxa and improve conservation practice.

The study was published in the peer reviewed journal "Science of the Total Environment" during the year 2017.

1.6 The current status of reptiles in legislation concerning the admission of plant protection products in the European Union

Regarding the placement of new PPP's in the European market, it is important to remark the current status of reptiles regarding ecotoxicological risk assessments. First of all, placement of

new PPP's is guided by Regulation 1107/2009 of the European Commission. Here, it's stated that the use of PPP's should have no unacceptable effects on the environment, whilst particularly focusing on effects on non-target species and organisms, including behavior, impact on biodiversity and the ecosystem. In essence, according to the current legislation, reptiles are in theory protected by law when talking about the placement of PPP's in the European market, being non-target organisms of pesticide applications. However, reptiles have traditionally not been included in risk assessments during admission procedures. In terrestrial testing, the main focus has lied on birds, mammals, bees, arthropods and plants. On the other hand, aquatic vertebrates (mainly fish and, in some cases, amphibians), algae, macrophytes and invertebrates have been the test subjects in the field of aquatic testing (van Leeuwen and Vermeire 2010, EFSA 2013). Generally speaking, reptiles have long been considered to be represented by birds and mammals in ecotoxicological studies (Weir et al. 2010). However, in the last few years, concerns have been raised regarding the question whether current risk assessment practice may not sufficiently cover the risk of reptiles.

These taxa display some major differences regarding physiology and ecology when compared to birds or mammals, which make them potentially more susceptible to effects of pesticide exposure. A study concerning the comparability between avian and reptile toxicant data revealed that birds were only more susceptible for 1/4 of all analyzed pesticides, for which comparable toxicological endpoints were available (Weir et al. 2010). This makes the use of birds as surrogate species difficult since, generally speaking, toxicological assessments should ideally be conducted using the most sensitive species (Sparling et al. 2010). The probably most important factor regarding differences in susceptibility is the fact that reptiles are poikilothermic organisms, while birds and mammals are homoeothermic (Gardner and Oberdörster 2006, Sparling et al. 2010). This condition influences sensitivity and chances of

exposure to pesticides, as it affects physiology, growth, development, behavior and reproduction, thus making comparisons between taxa rather difficult. It may further greatly influence potential exposure to pesticides. For example, biological activity in reptiles usually increases with higher temperatures, thus altering the food intake rate, which will influence oral exposure through contaminated prey. Similarly, metabolism is influenced by temperature, thus modifying toxicant metabolism. For instance, chemicals are more readily metabolized by more metabolically active organisms (i.e. homoeothermic organisms). At the same time, higher metabolic rates help lower the risks of suffering toxic effects at the physiological level (Talent 2005, EFSA 2017). In the case of reptiles, an increase in metabolism can only be guided by thermoregulation, accelerating physiological processes. Toxicant metabolism has thus an additional energy cost that can compromise other biological functions such as growth, development, immunity or reproduction (Talent 2005, Gardner and Oberdörster 2006). It has further been argued that poikilothermy may "protect" reptiles from toxicants which show an increase in toxicity in their metabolized state (as metabolites), as opposed to the "non-metabolized" state. However, the opposite is also possible (Weir et al. 2015).

A main aspect in reptile ecotoxicology is the great importance of the dermal exposure route whilst assessing contaminant uptake (Gardner and Oberdörster 2006, Hopkins 2006, Todd et al. 2010), as previously mentioned. This route of exposure is not considered in terrestrial risk assessments for any organisms during admission procedures of new PPP's (EFSA 2009), thus creating a very important data gap. In effect, this means that, as of now, no vertebrate species (neither birds nor mammals) can objectively be used as surrogate species to conduct risk assessments for reptiles, as one of the major, if not the most important, routes of exposure is not being considered, at all. Recent studies have elucidated the great importance of this exposure pathway regarding pesticide uptake (Weir et al. 2014, 2016).

In light of these uncertainties, a scientific opinion concerning the state of the science on pesticide risk assessment for amphibians and reptiles was made public during the year 2017, which should provide the scientific basis for potentially developing a guidance document for pesticide risk assessment for these taxa (EFSA 2017). Regarding reptiles however, the results of this scientific opinion are very clear: ecotoxicological data regarding effects of pesticide exposure is very scarce and, in most cases, not standardized. The authors conclude that currently, there is not sufficient data available to adequately assess the risks of pesticide exposure, and that further research and standardization is needed to be able to address this question. At the same time, they conclude that effects of pesticide exposure have been shown to have potentially severe consequences for individuals and populations, and that a specific risk assessment for this group of organisms is needed in order to preserve biodiversity and ensure that EC Directive 1107/2009 is complied with (i.e. “no unacceptable effects on the environment”, specifically, on non-target organisms).

1.7 Conclusion

Reptiles belong to a taxonomic group characterized by ongoing population declines. While there are many factors influencing this biodiversity loss, it is the scientific consens that, especially within industrialized countries, habitat loss and degradation, coupled with environmental pollution are the main driving factors for these declines. Environmental pollution in form of pesticides plays an increasingly important role here, due to the sheer amount and variety of different formulations that are applied each year. Nevertheless, reptiles are currently not subject of environmental risk assessments during the registration and admission of new PPP’s. Here, mammals and birds have been used as surrogate species up until now. However, due to the great differences in life-history, biology and ecology of these different taxa, the suitability of current risk assessment schemes is doubtful at best. Many

reptile species do regularily occur within agricultural habitats and are prone towards pesticide exposure, as our own research has shown. At the same time, we were able to verify effects of pesticide exposure regarding various endpoints, resulting in repercussions at the sub-individual, individual and population level. While the Common wall lizard is able to cope with observed effects relatively well, thanks to its population ecology and life history, the same may not be said for other reptile species. Considering that many reptile species within the EU, and all reptiles natively occurring in Germany are protected under Annex IV of the habitats directive, this gives a strong reason to act in order to improve conservation practice. Exposure of reptiles to pesticides has long been discussed, with different sources arguing that exposure may be minimal, and in any case covered by current risk assessments. Yet, our results showed that food items of the common wall lizard do indeed display a measurable pesticide load, representing a clear exposure route. Similarly, pesticide residues were found within deceased individuals, clearly demonstrating accumulation within reptile tissues. At the same time, dermal exposure has been argued to be the possibly most important uptake pathway of pesticides in reptiles. However, this scenario is not considered even in pesticide risk assessments for birds and mammals. Thus, it can be argued that the use of surrogate taxa, as currently practiced, is indeed unsitable. Our own research has shown that dermal exposure of Common wall lizards to different pesticide formulations resulted in similar sub-indivdual responses to that of orally exposed individuals. However, individual endpoints regarding locomotor performance and behavior were affected more strongly in dermally exposed individuals. In light of these findings, it seems indispensable to finally act, and for reptiles to be included in ecotoxicological risk assessments regarding PPP's, in order to improve conservation practice and minimize the effects of environmental contamination on global loss of reptile diversity.

In sight of the large data gaps concerning the protection of reptiles within the scope of ecotoxicological risk assessments and the lack of data in the field of reptile ecotoxicology, the aim of this thesis is to generate information regarding the exposure probability and risk of different reptile species, and to elucidate differences in sensitivity between different orders and suborders. Further, to establish new methods to detect these effects of pesticide exposure in reptiles, by ways of minimal invasive sampling methods, and to identify whether long-term exposed populations (several generations) suffer from effects of pesticide exposure at the sub-individual, individual and population level. It is the goal of this research that the gained data may in the future be used to establish and conduct environmental risk assessments and help protect and preserve reptile diversity.

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CHAPTER I

Risk evaluation of pesticide use to protected European reptile species

Norman Wagner[1], Valentin Mingo[1], Ulrich Schulte[1], Stefan Lötters[1]

[1] - Trier University, Department of Biogeography, Universitätsring 15, 54296 Trier, Germany

Highlights

- We evaluated different risks for protected European reptile species by pesticide use.
- Most species at high risk are threatened within their entire geographic ranges.
- All evaluated freshwater turtles are at high risk.
- Exposure risk within the conservation areas differs on a national scale.
- We suggest management plans to consider monitoring of habitat contamination.

Abstract

Environmental contamination is supposed to be a reason for population declines in reptiles. Especially intensification and expansion of agriculture are leading to increased pesticide exposure risks for wildlife. In the European Union, Special Areas of Conservation (SAC) have been established for the conservation of taxa listed in Annex II of the Habitats Directive. In the SAC, agricultural land use is legal. Therefore, we conducted a risk evaluation of pesticide exposure for Annex II reptiles by calculating proportions of land use with regular pesticide applications within SAC. Using three evaluation factors (occurrence probability, physiology, life-history aspects), a species-specific risk index was created. Most species at above-average risk by pesticide use are globally threatened with extinction (IUCN Red List of Threatened Species). About 25% of their SAC are agriculturally used and one priority subspecies of the Habitats Directive is at highest risk (Vipera ursinii rakosiensis). Also, all evaluated fresh-water and land-dwelling turtle species are at high risk. National variation in agricultural land use in the SAC was observed. Species at above-average risk are mainly distributed in the Mediterranean and Pannonian/Continental biogeographical regions of Europe. Conservation status according to the IUCN Red List of Threatened Species as well as national differences among the member states argue for the inclusion of pesticide risk assessments in site-specific management plans for SAC to avoid regional loss of reptilian biodiversity.

Introduction

Biodiversity decline is a serious and widely recognized problem among all taxa and ecosystems over the entire globe. In reptiles, worldwide population declines have been noted (Gibbons et al., 2000). A first analysis of their global conservation status revealed that nearly one in five reptilian species is threatened with extinction, while for another one in five information is lacking (Böhm et al., 2013). The causes for declines are assorted. For 'industrialized' countries, habitat loss and degradation are most extensively contributing to population declines (Todd et al., 2010). In these countries, primary and secondary reptile habitats have been transformed into areas of intensive agricultural land use. As a spin-off, species additionally become more and more exposed to agrochemicals, especially pesticides (Weir et al., 2010).

Today, massive land use change can be observed in Europe, for instance, related to the growing impact from energy crops (Fargione et al., 2010). Additionally, there is a trend to grow energy crops on previously uncultivated land including former mining areas (Dauber et al., 2012). Such areas are known to serve as crucial secondary habitats for reptiles (Günther, 1996; Böhme et al., 1999). In the future, the cultivation of genetically engineered crops – which are created to stand adverse abiotic conditions like too low soil pH – might even increase the inclusion of previously non-arable areas (Pengue, 2005). It is no surprise that solely in Europe, 18 % of all reptile species are listed as threatened with extinction (Cox and Temple, 2009; Böhm et al., 2013).

The contribution of environmental contaminants, especially pesticides, to reptile declines has yet been little addressed. Even with regard to simple acute toxic effects only marginal information is available, although showing its importance. As an example,in Hermann's

tortoises (*Testudo hermanni*) from southern Greece, a significantly reduced survival and symptoms of poisoning after herbicide applications was reported (Willemsen and Hailey, 2001). Evidence of potentially strong impacts on European reptile wildlife has been linked to sublethal concentrations. Wall lizards (*Podarcis bocagei*) from Portugal, for instance, revealed an increase of hemoparasites, reduced liver size, lack of energetic reserve accumulation, oxidative stress, increased thyroid activity, disturbance of sex ratio and general loss of fitness after pesticide exposure (Amaral et al., 2012a,b,c; Bicho et al., 2013). In the Americas, white blood cell counts decreased in *Caiman latirostris* due to herbicide contamination (Latorre et al., 2013), while laboratory and field studies detected a depressed clutch viability, reduced neonatal survival, hermaphroditism, and reduced testosterone concentration, i.e. endocrine disruption, in another crocodilian, *Alligator mississippiensis* (Guillette et al., 1994; Crain et al., 1997). Pesticide uptake in reptiles is supposed to be mainly via the food chain (Weir et al., 2010). Herbivorous and omnivorous species may suffer from direct ingestion of pesticides sprayed on plant surfaces, while in carnivorous and omnivorous reptiles biomagnification may play an important role (Biddinger and Gloss, 1984). In relation to nutrition, physiology influences pesticide uptake. Species with small body indices show a much greater increase in dietary exposure when compared to individuals of larger species (Weir et al., 2010). Another pathway of pesticide absorption in reptiles is dermal uptake from the contaminated environment (Hopkins, 2005). Again, a small body size means a greater contact surface relative to the body mass, promoting a comparatively higher uptake of pesticides (Murphy and Murphy, 1971). Dermal uptake in squamate reptiles also depends on pholidosis (Chang et al., 2009) as well as the lipid and keratinocyte composition of the skin (Roberts and Lillywhite, 1980; Palmer, 2000; Toni et al., 2007). Lastly, life-history aspects play an important role in reptilian pesticide exposure and uptake. Species with relatively small home ranges and migration rates can be highly threatened by the regionally intensive use of

pesticides, as the ability for them to leave an exposure area is low. Conversely, species with larger home ranges may be more likely to come in contact with pesticides due to wide-ranging behavior (Günther, 1996; Böhme et al., 1999; Southwood and Avens, 2010). Furthermore, populations of species with relatively few offspring and species that need longer time to reach sexual maturity (K-strategists) will suffer more intensively from effects on individuals than r-strategists (Pianka, 1970).

In general, various problems arising from land use conflicts – including mechanical and chemical intensification of agriculture – are affecting protected areas (Jetz et al., 2007). With the Habitats Directive 92-43-EEC of the European Union (EU, 1992), the European Council set up the Natura 2000 network, which is "a coherent European ecological network of special areas of conservation" (EU, 1992). The goal of the Natura 2000 network is to assure the long-term conservation of Europe's natural heritage (threatened species and habitats, which are listed in different annexes), thus fulfilling a Community obligation under the UN Convention on Biological Diversity (http://ec.europa.eu/). Although the Habitats Directive has been criticized, among others, for the lack of flexibility concerning fixed lists of protected species (Hochkirch et al., 2013) or insufficient consideration of optimal site designation and management (Gaston et al., 2008), this network is considered as one of the largest and most important conservation networks of the whole world (Lockwood, 2006). The Natura 2000 network is comprised of 'Special Areas of Conservation' (SAC) designated by member states under the Habitats Directive (and also incorporates special protection areas, which they designate under the European Birds Directive) (http://ec.europa.eu/).

There have been three stages in the selection of SAC. (1) The member states carried out assessments on habitat types listed in Annex I and species occurrence listed in Annex II of the Habitats Directive to choose national sites. Annex II lists species which are of community

interest and whose conservation requires the designation of ‘Special Areas of Conservation’ (SAC) (EU, 1992).

With regard to reptiles, 21 species and 3 subspecies are listed in Annex II. Seven are ‘priority species’ of the Natura 2000 network; these require an enhanced protection status (Table 1). (2) On the basis of national lists, the European Commission adopted a list of sites of community importance, in agreement with the member states including interests of relevant stakeholders, land owners and users, and environmental NGOs. (3) Based in the list of sites of community importance, the member states designated the SAC. The member states must take the necessary management or restoration measures within SACs to ensure the favorable conservation status of species and habitats within the biogeographical regions of Europe including regular monitoring and management plans (http://ec.europa.eu/).

The Natura 2000 network shall not be a system of strict nature reserves where all human activities are excluded. Most of the land is privately owned with the emphasis that future management is sustainable, both ecologically and economically (http://ec.europa.eu/). Hence, agricultural land use does not stop at SAC borders and at defined conditions land use within them is possible (EU, 1992).

Due to the aforementioned conservation requirements for protecting reptile diversity and the potential threats to them from pesticide use, it is crucial to test if current land use practice with regular pesticide applications is likely to affect reptiles within their SAC. With the purpose to test this, we conduct a spatial risk evaluation at the European level. Commonly, a toxicity risk assessment is divided into four steps: (1) hazard identification, (2) exposure assessment, (3) effects assessment and (4) risk characterization (Van Leeuwen, 2007). Number one can be seen as a first screening step. What differentiates risk from hazard is the

likelihood of harm due to exposure. Exposure assessment comprises the measuring of exposure concentrations (here: pesticides in general), once chemicals are produced, used and emitted. Effects assessment (also known as dose-response-assessment) is the estimation of the relationship between dose or level of exposure to a substance, and the incidence and severity of an effect (here: to reptiles). Finally, the risk characterization is the estimation if adverse effects are likely to occur in a population or environmental compartment. This integrates the first three steps (US EPA 1986; Van Leeuwen, 2007). Number one can be seen as a first screening step. What differentiates risk from hazard is the likelihood of harm due to exposure. Exposure assessment comprises the measuring of exposure concentrations (here: pesticides in general), once chemicals are produced, used and emitted. Effect assessment (also known as dose–response-assessment) is the estimation of the relationship between dose or level of exposure to a substance, and the incidence and severity of an effect (here: to reptiles). Finally, the risk characterization is the estimation if adverse effects are likely to occur in a population or environmental compartment. This integrates the first three steps (US EPA, 1986; Van Leeuwen, 2007).

Up to now, reptiles have been understudied in ecotoxicology (Köhler and Triebskorn, 2013; Weir et al., 2015), i.e. not only specific laboratory data but especially data on causative relationships between pesticide use and reptile population declines are yet lacking. Therefore, detailed risk assessments on European reptile species are not possible yet and our risk evaluation should be regarded as the first attempt to contribute to the first two steps of a risk assessment (i.e., hazard identification and exposure assessment). Only combined with new data from the laboratory (or mesocosms), our results could be used to conduct an actual risk characterization and thus execute an actual risk assessment for the here reviewed reptile species.

In the present study, we evaluate three different risks for European reptile species: (1) potential exposure, (2) potential individual sensitivity and (3) potential vulnerability of their populations. For this purpose, we identify the "proportions of land use with regular pesticide applications" (%LPA) within SAC that were created for Annex II reptiles and combine this spatial data with evaluation factors of species' ecology, physiology and biology.

Methods

Land use with regular pesticide applications within the SAC ("potential exposure")

We calculated %LPA within SAC that were created for Annex II reptile species using ArcMap 10 (Esri®) and the latest version (2006, updated 2011) of the European CORINE (Coordination of Information on the Environment) land cover data. CORINE data and those for Natura 2000 sites and species were obtained from the European Environmental Agency (http://eea.europa.eu). In the CORINE project, mapping of the land cover was performed on the basis of satellite remote sensing images on the scale 1:100,000. Agricultural land cover classes (under the CORINE-Label "agricultural areas"), which reflect areas where pesticides are regularly applied, were chosen, these were CORINE land cover classes 211 ("non-irrigated arable land"), 212 ("permanently irrigated land"), 213 ("rice fields"), 221 ("vineyards"), 222 ("fruit trees and berry plantations"), 223 ("olive groves"), 241 ("annual crops associated with permanent crops"), 242 ("complex cultivation patterns"), 243 ("land principally occupied by agriculture with significant areas of natural vegetation") and 244 ("agro-forestry areas"). We are aware that cultivation and pesticide use practices differ between and in these classes (often annually), but more detailed information is not available for the entire EU. Although we realize that on intensively used hay meadows pesticides are regularly applied, we excluded land cover class 231 ("pastures") because it is not possible to

distinguish between those pastures and real pastures. Conversely, parts of the European agricultural area are organic (see Discussion). Since no actual land cover data were available for Greece and the UK, these countries were excluded from the evaluation.

Species Risk Indices and Pesticide Risk Factors

Not only habitat exposure but also life-history traits and physiology of the considered reptile species (Table 1) remarkably differ, we created a species risk index (SRI) for each taxon reflecting its potential general risk based on literature data and – when possible – presence/absence data (coordinates from the Global Biodiversity Information Facility, GBIF (http://data.gbif.org) and HerpNET (http://www.herpnet.org) for occurrence data and pseudo absence data; see below for details). Three evaluation factors (EFs) for exposure risk were considered to define the SRI. The SRI combined with %LPA defined the species' pesticide risk factor (PRF).

Evaluation Factor for habitat exposure risk (EF 1) ("potential exposure")

Together with the spatial data on agricultural land use, EF 1 refers to the potential "exposure risk" of a species. For EF 1, we awarded 1 Risk Point (RP) when habitat exposure risk was 'high' and 0 when it was 'low'. In a first step, information was obtained from the literature (Gasc et al., 1997; Böhme et al., 1999; Cox and Temple, 2009) and from the IUCN Red List of Threatened Species (http://www.iucnredlist.org). The literature-based estimates of habitat exposure are given in Appendix A.

For 11 species and subspecies (see Appendix A), the literature-based estimates were used for evaluating their habitat exposure risk. For the remaining nine taxa, sufficient occurrence data were available to use logistic regression models to predict presence/absence as a function of

%LPA. When the presence of a species positively correlated with %LPA, a regular occurrence in cultivated landscapes was suggested. Hence, 1 RP was awarded. 0 RP was given if there was no significant trend, so that it can be suggested that species usually do not occur within cultivated landscapes. Occurrence data were corrected for duplicates and implausible records (e.g. records far outside of a species native range). For species with ≥ 100 records (n = 5), we randomly chose a subset of 100 localities, respectively. For species with less than 100 but more than 10 records (n = 4), we considered all records as 10 is the minimum sample size per predictor (here: %LPA) in logistic regressions (Agresti, 2007).

We set a 1 km-buffer around each presence record to account for potential migration and dispersal. We are aware that distances of both home ranges and dispersal capacities can remarkably differ among species and even within populations depending on habitat types and connectivity. However, 1 km is acceptable as an average maximum range (Günther, 1996; Böhme et al., 1999). Because of concern on spatial autocorrelation, presence records had to be at least 2 km apart to ensure that the 1 km circles do not overlap. Consequently, species with less than 10 suitable presence points (i.e. whose 1 km buffers do not overlap) were not considered in further analyses (n = 11).

In a subsequent step, for each species, absence points were created in equal numbers to the presence points, respectively. For this purpose, we used a random sample of locations from SAC within the species' distribution range (http://www.iucnredlist.org), but where the considered species was not listed. Also absence points had to be at least 2 km apart and 1 km buffers were set. Finally, as a predictor for the presence/absence of a species, the %LPA was calculated within all buffers. Spatial data were processed using ArcMap 10. All statistical analyses were performed with the *R* and the *MASS* package (R Developmental Core Team, Vienna).

Evaluation Factor for species' physiology (EF 2) ("potential individual sensitivity")

This EF refers to the "potential individual sensitivity" caused by pesticide use. As mentioned in the Introduction, species with small body indices show a much greater increase in dietary exposure when compared to individuals of larger species (Weir et al., 2010) and greater contact surface promoting a respectively higher uptake of pesticides (Murphy and Murphy, 1971). Therefore, we took the average snout-vent-length (and carapax length for turtles respectively) as a proxy to account for species' differences concerning their different physiology (taken from the literature; Appendix A). We estimated the probability distribution of the data (i.e. a quantitative variable) using the histogram function in R. The data were classified into five classes. Hence, for EF 2 we awarded 0 to 4 RPs.

Evaluation Factor for life-history (EF 3) ("potential vulnerability of populations")

This EF refers to the "potential vulnerability of populations" caused by pesticide use. Reptile species with a K-strategy, that is (1) with relatively few offspring (clutch size, hatchlings), (2) with low reproductive (clutch) frequency per year and (3) when longer time is needed to reach sexual maturity are supposed to suffer more from effects on individuals than r-strategists.We considered these three life-history aspects for reproductive potential by classifying (1) the average clutch/offspring size, (2) clutch frequency per year and (3) time to reach sexualmaturity.

Again, all data were literature-based (Appendix B). Data were grouped into four to seven classes (4 classes for average clutch frequency/year (0–3 RPs), 6 classes for average clutch/offspring size/year (0–5 RPs) and 7 classes average time to reach sexual maturity (0–6 RPs)). Hence, according to our classification, a species could score a maximum of 14 RPs for EF 3.

Calculation of the Pesticide Risk Factors (PRF)

Employing EFs 1–3, a species could maximally receive 19 RPs (cf. Appendices A–C). In a first step, the sum of the RP defined the SRI for each taxon. Based on the SRI and the %LPA within a species' SAC, we eventually calculated the PRF using a modified formula under which a species habitat can score PRFs 0–1 (Wagner et al., 2014).

PRF = SRI * %LPA / 19 * 100

(SRI = sum of awarded RP; 19 = maximum points that could be awarded)

Because of concerns about the robustness of our evaluation to changes in the definition, scale or number of categories, we additionally gave equal weights for the three EF by converting the awarded RP to a relative scale of 0-10, so that a species at maximum could score here 10 points for habitat, 10 points for physiology and 10 points for life-history (see Appendices A-C). The formula was changed to

$PRF_{weighted}$ = SRIweighted * %LPA / 30 * 100

($SRI_{weighted}$ = sum of weighted RP; 30 = maximum points that could be awarded after weighting)

Finally, PRF and $PRF_{weighted}$ were compared using Wilcoxon signed rank tests with continuity correction.

National variation

To demonstrate national variation in risk by pesticide use for Annex II reptiles, we additionally calculated %LPA within national SAC for all species, which are distributed in

more than one EU member state. We tested if %LPA and thereby risk significantly differs between member states. Therefore, the %LPA within the national SAC of a species were compared. For all comparisons, one-way ANOVA followed by Bonferroni-corrected post-hoc tests were conducted (some data had to be Box-Cox-transformed prior to analysis).

Results

%LPA within the SAC and Evaluation Factors

The average (current) %LPA within the SAC was 14.37% (± 2.71) and ranged from less than 1% (0.07 ± 0.04 km²) in SAC that were created for the Pyrenean rock lizard (*Iberolacerta bonnali*) to more than 45 % in SAC for the subspecies *rakosiensis* of the Meadow viper (*Vipera ursinii*) (201.84 ± 60.60 km²) (Table 1; Figure 1; Appendix C). In the SAC of ten taxa, the %LPA was above-average (about 15-45%; Table 1).

As mentioned, the data for EF 2 (species' physiology, i.e. average snout-vent-length) were grouped into five classes (0-20, > 20-40, > 40-60, > 60-80 and 120-140 cm). Most taxa (14) were classified into the first group (0-20 cm) and, therefore, received 4 RP for EF 2. Three taxa received 2-3 RP, while the large European colubrid snakes *Hierophis cypriensis*, *Natrix n. cypriaca* and *Elaphe quatuorlineata* only received 1 and 0 RP (Appendix A).

For EF 3 (life-history), species received RP for three different factors: (1) "Average clutch size/number of offspring", (2) "Average reproductions/year" (i.e., number of clutches/offspring per year) and (3) "Average time to reach sexual maturity". Information was literature-based (Appendix B).

(1) "Average clutch size/number of offspring" was grouped into six classes (2-4, > 4-6, > 6-8, > 8-10, > 10-12 and 14-16). *Iberolacerta bonnali* and *Euleptes europaea* have the smallest

clutch sizes and, therefore, received the highest RP (5). Nine species only produce an average of 4-6 eggs and received 4 RPs, seven taxa produce 6-10 eggs/offspring and received 2-3 RP, while *Natrix n. cypriaca* and *Elaphe quatuorlineata* have the largest clutch sizes (Appendix B).

(2) "Average reproductions/year" was grouped into four classes (1-1.5, > 1.5-2, > 2-2.5 and > 2.5-3). Eighteen taxa received 2-3 RP because they only reproduce on average 1-2 times per year, but *Testudo hermanni* and *Podarcis lilfordi* 2-3 times (Appendix B).

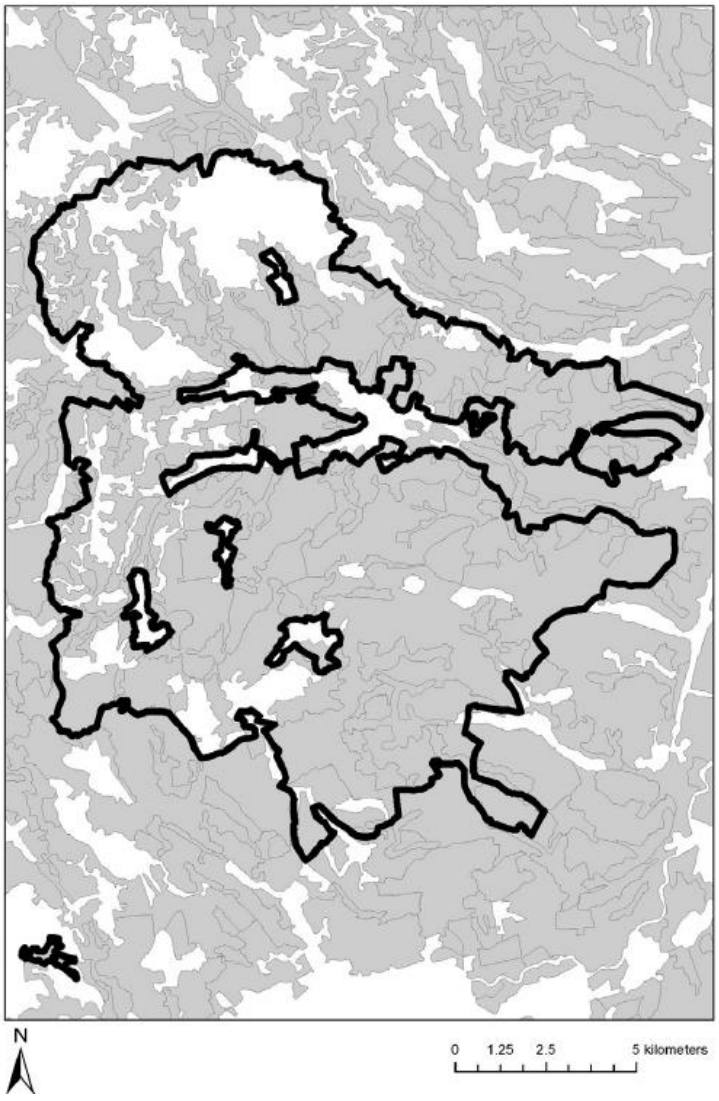

Figure 1: Example of high agricultural land use within the Romanian Special Area of Conservation 'Dealurile Clujului de Est' (ROSCI0295), which was (among other Annex II species) created for the reptiles *Vipera ursinii rakosiensis* and *Emys orbicularis*. The borders of the protected area are black surrounded; the gray polygons indicate land cover classes where pesticides are regularly applied.

(3) “Average time to reach sexual maturity” was grouped into seven classes (> 8-9, > 7-8, > 6-7, > 5-6, > 4-5, > 3-4 and 2-3 years). The turtles *Testudo graeca, T. hermanni* and *T. marginata* received 6-5 RP because they need on average 7-8.5 years to reach sexual maturity. *Emys orbicularis, Mauremys leprosa* and the two snakes *Hierophis cypriensis* and *Elaphe quatuorlineata* have an intermediate time span, while the remaining 13 taxa quickly reach sexual maturity (Appendix B).

Species Risk Indices and Pesticide Risk Factors

The awarded RP amounted to the SRI,which were in average 11.1 ± 0.6 (Table 1). The turtles T. graeca, T. hermanni and E. orbicularis revealed the highest, P. lilfordi and the two snakes E. quatuorlineata and N. n. cypriaca the lowest SRI (Table 1).

Table 1: Categories under the IUCN Red List of Threatened Species, “proportional Land use with regular Pesticide Applications” (%LPA) within “Special Areas of Conservation” (SAC), Species Risk Indices (SRI) and Pesticide Risk Factors (PRF) of Annex II reptiles. Above-average values are in bold.

IUCN status[a]	**%LPA**[b] **within SAC**	**SRI**	**PRF**
Critically Endangered			
*Gallotia simonyi**	1.34 %	11	0.01
Endangered			
Podarcis lilfordi	3.64 %	8	0.02
Chalcides simonyi	3.91 %	11	0.02
*Hierophis (Coluber) cypriensis**	1.20 %	10	0.01
*Vipera ursinii rakosiensis**[c]	45.12 %	10	**0.24**
Vulnerable			
Testudo graeca	18.00 %	17	**0.16**
Mauremys caspica[d]	30.02 %	10	**0.16**
Mauremys leprosa[d]	26.84 %	12	**0.17**
Iberolacerta (Lacerta) monticola	7.29 %	11	0.04
Vipera ursinii	7.59 %	10	0.04
Near Threatened			
Testudo hermanni	21.87 %	14	**0.16**
Emys orbicularis	23.36 %	14	**0.17**
Iberolacerta (Lacerta) bonnali	0.19 %	13	0.00

Lacerta schreiberi	15.50 %	13	**0.11**
Podarcis pityusensis	5.14 %	11	0.03
Euleptes europaea	9.08 %	12	0.06
Elaphe quatuorlineata	23.60 %	7	**0.09**
Least Concern			
Testudo marginata	16.28 %	12	**0.10**
Gallotia galloti insulanagae[e]	6.13 %	12	0.04
Zamenis (Elaphe) situla	27.69 %	11	**0.16**
*Natrix natrix cypriaca**[f]	6.84 %	5	0.02
			Ø 0.09

* = priority species
[a] = The marine turtles *Caretta caretta* and *Chelonia mydas*, which are European priority species, have not been evaluated. Also the priority species *Macrovipera schweizeri* could not been evaluated due to lack of actual land cover data from Greece.
[b] = Excluding Greece and the UK due to the lack of land cover data.
[c] = *Vipera ursinii rakosiensis* is still listed for the Natura 2000 site 'AT1220000' but already extinct in Austria why this site was excluded.
[d] = *Mauremys leprosa* not assessed by the IUCN but by Cox and Temple (2009); *M. caspica* as part of *M. leprosa*.
[e] = no specific IUCN assessment for this subspecies, but *Gallotia gallotia insulanagae* is considered Near Threatened by the national Spanish Red List.
[f] = no specific assessment for this subspecies

Using the described formulae, the SRI or SRIweighted and the current %LPA defined the final "pesticide risk factor" (PRF) or $PRF_{weighted}$ of a species. PRF and $PRF_{weighted}$ did not change or only little (from 0 to ± 0.07; Table 2). Species at above-average risk stayed the same (with one exception: Lacerta schreiberi; cf. Tables 1 and 2 and see Discussion), all changes were not significant (V = 99.5, p = 0.85) and thereforewe regarded the PRF (based on the sumof RP=SRI) as robust.

Table 2: Species risk indices (SRIweighted) after converting the Risk Points (RPs) of each evaluation factor EF) to a relative scale of 0–10 to test for robustness of the PRF. Pesticide risk factors (PRFs) and $PRF_{weighted}$ did not significantly differ (p N 0.05). Above-average $PRF_{weighted}$ are in bold.

Species	**SRIweighted**	**PRFweighted**
Gallotia simonyi[a]	15	0,01
Podarcis lilfordi	12,86	0,02
Chalcides simonyi	15	0,02
Hierophis (Coluber) cypriensis[a]	8,93	0
Vipera ursinii rakosiensis[a]	21,79	**0,33**
Testudo graeca	28,57	**0,17**
Mauremys caspica	23,57	**0,24**

Mauremys leprosa	25	**0,22**
Iberolacerta (Lacerta) monticola	15	0,04
Vipera ursinii	21,79	0,06
Testudo hermanni	26,43	**0,19**
Emys orbicularis	26,43	**0,21**
Iberolacerta (Lacerta) bonnali	16,43	0
Lacerta schreiberi	15,71	0,08
Podarcis pityusensis	25	0,04
Euleptes europaea (Phyllodactylus europaeus)	15,71	0,05
Elaphe quatuorlineata	14,29	**0,11**
Testudo marginata	23,21	**0,13**
Gallotia galloti insulanagae	15,71	0,03
Zamenis (Elaphe) situla	20,71	**0,19**
Natrix natrix cypriaca[a]	5,36	0,01
		Ø 0.10

a = priority species.

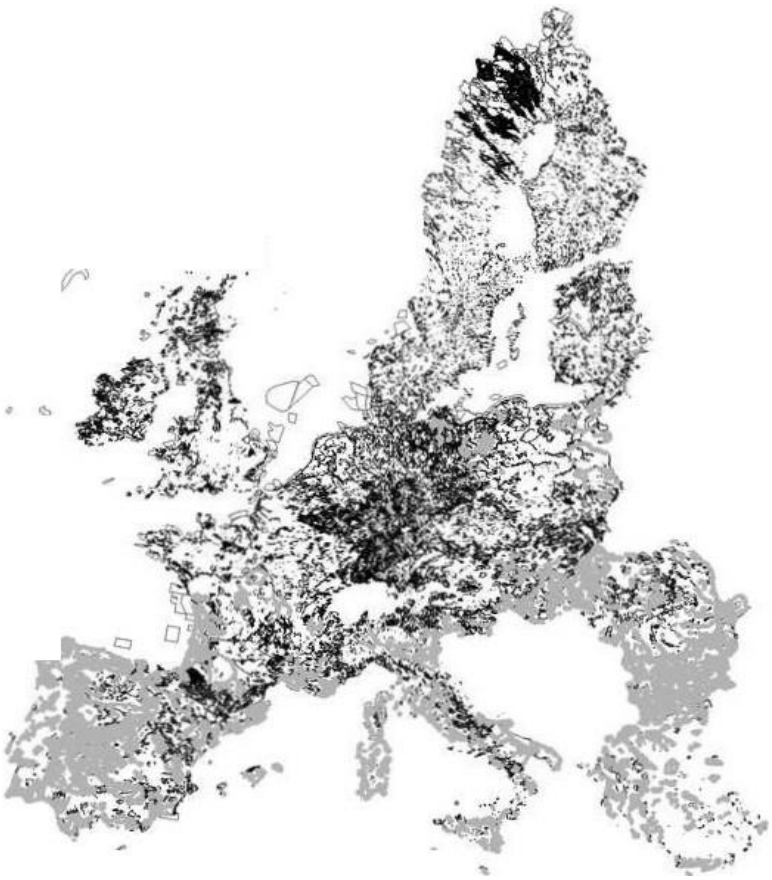

Figure 2: In the Natura 2000 network of protected areas, most Special Areas of Conservation (all polygons represent SAC) that were created for Annex II reptile species at above-average risk by pesticide use (gray polygons) are situated in the southern parts (Mediterranean region) or the south-eastern parts (Pannonian and Continental region) of Europe compared to others (black surrounded polygons). Note that the Azores, the Canary Islands and Cyprus are excluded for better graphic representation.

Ten species – including all six Annex II turtles – are at above-average risk by pesticide usewithin their SACs. In six cases, the high PRF resulted due to both high proportions of agricultural land use within the SACs and high sensitivity based on physiological and life-history aspects (i.e. high SRI). Conversely, the above-average PRF of four species (*Mauremys caspica, Zamenis situla, Vipera u. rakosiensis* and *E. quatuorlineata*) were mainly based on high proportions of agricultural land use (Table 1; Appendices A–C). Most species at aboveaverage risk occur in the southern and south-eastern parts of Europe (Fig. 2), which represent Mediterranean and Pannonian/Continental biogeographical regions, respectively, which are also known for their high reptile species richness (Gasc et al., 1997).

Priority species and global conservation status

With regard to the seven European priority species, the two marine turtle species have not been evaluated. Moreover, *Macrovipera schweizeri* could not been evaluated due to lack of actual land cover data fromGreece. From the remaining four priority species, *Gallotia simonyi, H. cypriensis* and *N. natrix cypriaca* are at low risk (PRFs 0.01–0.02; Table 1) within their SACs, whereas over 45% of the SACs that were created for *V. ursinii rakosiensis* are currently agriculturally used and, consequently, the highest PRF (0.32; Table 1) was assigned to this taxon.

Regarding the threat of species within their entire range and on the basis of the IUCN Red List of Threatened Species, out of the ten reptile specieswith above-average PRF, four are listed as Vulnerable or even Endangered, but only four as Near Threatened and two as Least Concern (Table 1).

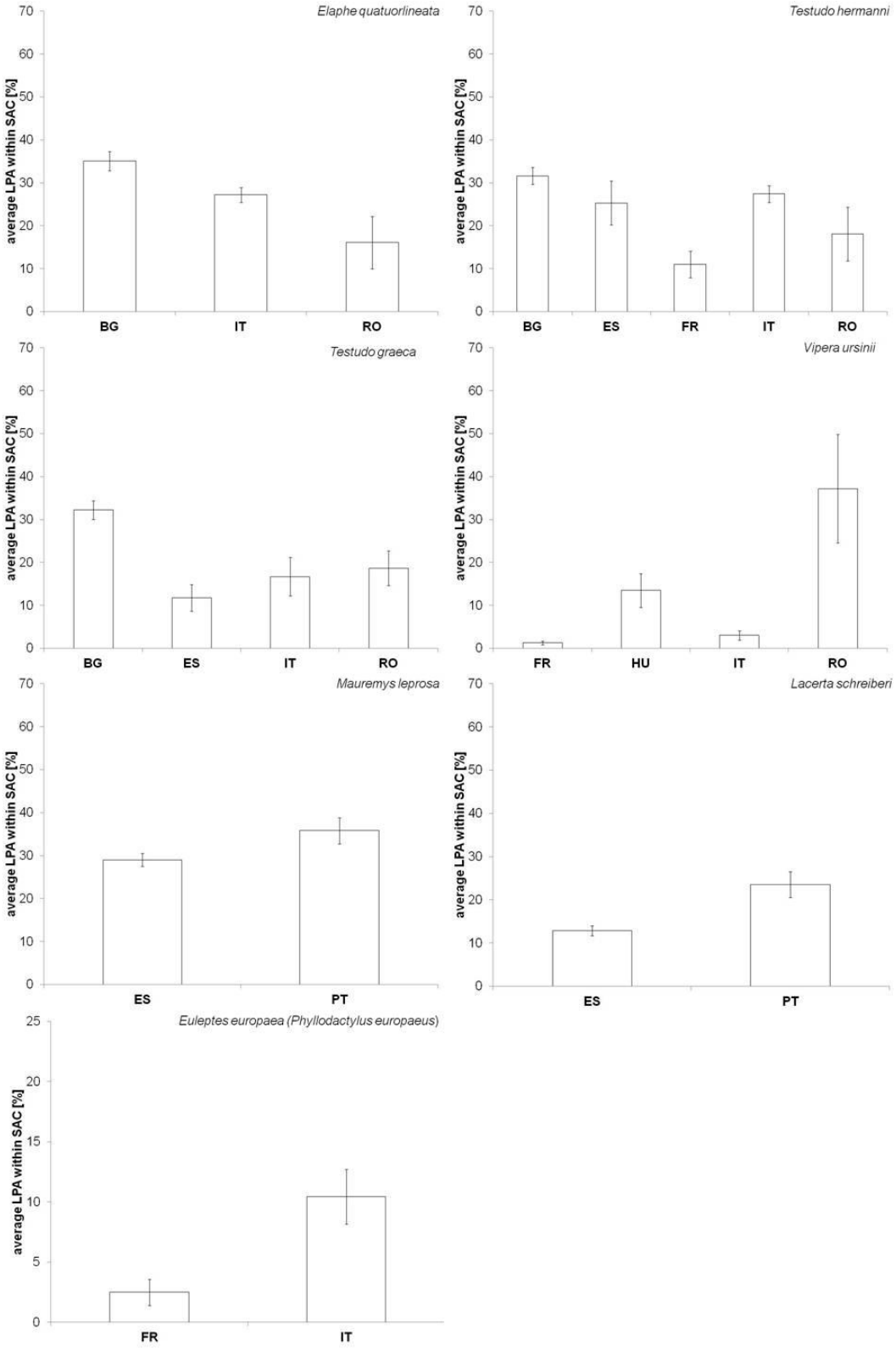

Figure 3: National variations of "proportional Land use with regular Pesticide Applications" (%LPA) (± SE) within Special Areas of Conservation (SAC) that were created for *Elaphe quatuorlineata, Testudo hermanni, T. graeca*, *Vipera ursinii*, *Mauremys leprosa*, *Lacerta schreiberi* and *Euleptes europaea*. Abbreviations: BG = Bulgaria; ES = Spain; FR = France; HU = Hungary; IT = Italy; PT = Portugal; RO = Romania.

Variations at the national scale

Seven out of 12 species, which occur in more than one EU member states, have significant differences of %LPA within their national SAC (Figure 3; Appendices D-E). Especially in Bulgarian and Portuguese SAC that were created for Annex II reptiles, high proportions of LPA could be identified. This also accounts for Italian SAC, but only for certain species. Finally, Romanian SAC have usually low LPA, but the SAC that were created for *Vipera ursinii* are nearly half-covered by %LPA (Figure 3).

Discussion

According to our study, all six turtle species that are listed in Annex II of the Habitats Directive are at high risk by the use of pesticides. This is based on all three evaluated risks (exposure, sensitivity, vulnerability) and these turtles additionally show high proportions of agricultural land use within their SAC (about 16–30%; Table 1). Particularly, turtles need relatively long timeto their first reproduction and, in consequence, adverse (long-term) effects of pesticide use on individuals might result in stronger effects at the population level compared to species reaching sexual maturity faster (Pianka, 1970). Considering our spatial risk evaluation as part of a first step hazard identification together with conducted laboratory/mesocosm studies, T. hermanni is one of the few European reptiles forwhich toxicological tests have revealed sensitivity to herbicide use (Willemsen and Hailey, 2001). Four out of ten species at above-average risk are listed as at least Vulnerable by the IUCN Red List of Threatened Species. However, most Annex II reptiles are endangered within their entire ranges or are listed as Near Threatened and only four of the evaluated 21 Annex II reptiles are listed as Least Concern by the IUCN (Cox and Temple, 2009). Thismight be the reason for the high proportion of endangered species at above-average risk. Contrariwise,

Wagner et al. (2014), in their amphibian study, found that most Annex II taxa at above-average risk were listed Least Concern. But in the case of Annex II amphibians there is an inverse relationship between risk and conservation status: nearly half of them are listed as Least Concern by the IUCN (http://www.iucnredlist.org).

The significant differences between proportions of LPA within national SAC strongly argue for species- and site-specific evaluations to avoid regional loss of reptilian biodiversity. Site-specific detailed evaluations of pesticide contamination should start in the EU member states, which reveal the highest %LPA in the SAC of their Annex II reptiles. Mainly, these are member states from the southern (Mediterranean) or south-eastern (Pannonian/Continental) regions of Europe (Fig. 2), generally known for their higher reptile species richness compared to the rest of Europe (Gasc et al., 1997). Evaluations should include detailed information on species occurrence, population fluctuations, cultivation and pesticide application practices to possibly link reptile population declines with increasing pesticide use or use of specific formulations. With such data, the final steps of a risk assessment could be conducted (US EPA, 1986; Van Leeuwen, 2007).

Robustness and limitations of our first attempt of a risk evaluation

Concerning the robustness of our first attempt of a risk evaluation forEuropean reptiles to changes in the definition, scale or number of categories, we regarded the PRF (based on the simple sum of RP) as valid because no or no statistically significant differences between PRF and PRFweighted were observed. However, this considered robustness may change if a species score relatively high by having the size and reproductive mode considered ‘risky’ even though its habitat is mostly remote from agriculture and agrochemical exposures. With regard to the Annex II reptile species, this is only the case for L. schreiberi (cf. Appendices

A–C) and all remaining species at high risk usually occur in agriculturally used areas. However, when considering a wider range of species, this problem has to be taken into account. Conversely, pesticide drift into mountainous habitats far away from the application area has been observed in several studies (Sparling et al., 2001; Davidson et al., 2002; Davidson, 2004; Fellers et al., 2004; Davidson and Knapp, 2007).

Finally, to conduct a complete risk assessment at the European level, data on (i) detailed pesticide use, (ii) habitat contamination and effects on reptiles at the (iii) individual and especially (iv) at the population level are necessary. Such data could be obtainedwhen specificmonitoring programs will be part of the management plans of SAC. Such monitoring action should at least be considered for SAC, which are under high land use pressure and were created for Annex II reptiles that are threatened within their entire territories.

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Risk of pesticide exposure for reptile species in the European Union

Valentin Mingo[1], Stefan Lötters[1], and Norman Wagner[1]

Trier University, Department of Biogeography, Universitätsring 15, 54296 Trier, Germany

Highlights

- At least one third of all European reptile species have an increased pesticide exposure risk
- Two of them are threatened with extinction in the IUCN Red List of Threatened Species
- Members of the suborder lacertilia are apparently the most sensitive reptiles
- Species in southern countries are likely to suffer more due to multiple pesticide applications

Abstract

Environmental pollution has an especially high impact on wildlife. This is especially the case in industrialized countries. Although, many species within the European Union benefit from protection by the Habitats Directive, no special consideration is given to possible detrimental effects of pesticides. This is in particular remarkable as negative effects, which may lead to a regional diversity loss, have already been identified in laboratory and mesocosm studies. We conducted a pesticide exposure risk evaluation for all European reptile species with sufficient literature data on the considered biological and ecological aspects and occurrence data within agricultural areas with regular pesticide applications (102 out of 141). By using three evaluation factors – (i) pesticide exposure, (ii) physiology and (iii) life history – a taxon-specific pesticide exposure risk factor (ERF) was created. The results suggest that about half of all evaluated species, and thus at least 1/3 of all European species exhibited a high exposure risk. At the same time, two of them (*Mauremys leprosa* and *Testudo graeca*) are globally classified as threatened with extinction in the IUCN Red List of Threatened Species. Variation regarding species occurrence in exposed landscapes between pesticide admission zones within the EU is rather large. This variation is mainly caused by differing land use and species abundances between zones. At the taxonomic level, significant differences in exposure risk can be observed between threatened and non-threatened species, which can be explained by the formers remote distribution areas. Lizards display the highest sensitivity toward pesticides, although no differences in overall ERFs can be observed between taxonomic groups.

By identifying species at above-average risk to pesticide exposure, species-based risk evaluations can improve conservation actions for reptiles from cultivated landscapes.

Capsule Abstract

In this work, we evaluated the exposure risk of European reptile species towards pesticides and conclude, that at least one third of all species show an increased exposure risk.

Introduction

Biodiversity decline is a global problem among all taxa and ecosystems (Stuart et al., 2010). In reptiles, as one of the various threatened vertebrate groups, ongoing worldwide population declines are recognized (Amaral et al., 2012a; Gibbons et al., 2000; Todd et al., 2010). The causes for these declines are highly assorted, and it is believed that among all factors, habitat loss and degradation is the major factor in industrialized countries (Gibbons et al., 2000; Todd et al., 2010), followed by agrochemical use in their habitats (Bicho et al., 2013; Gibbons et al., 2000; Todd et al., 2010; Weir et al., 2010). 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; IUCN, 2015).

Although effects of pesticides on reptiles have been reviewed to some degree, and different studies have shown evidence of potential strong effects on reptile wildlife (Cardone, 2015; Carpenter et al., 2016; Douros et al., 2015; Latorre et al., 2013; Poletta et al., 2016; Schaumburg et al., 2015; Weir et al., 2010, 2014, 2015; Willemsen and Hailey, 2001), there is still a great lack of data. Especially, toxicity data concerning squamates is scarce (Sparling et al., 2010; Weir et al., 2010), and data on effects of pesticides in species' natural habitats even more so. Additionally, reptiles are currently not considered for risk evaluation processes during pesticide admission procedures in the EU (EFSA, 2009; Regulation (EC) No 1107/2009). While knowing the potential effects of pesticides on reptile populations is indeed

of great importance, knowing which reptile species and populations may come into contact with pesticides in the first place seems of equal significance. This approach would allow us to identify which species will suffer the most due to pesticide use, and could thus provide key data for conservation practice.

So far, there have been few studies concerning herpetological biodiversity patterns within different croplands (Balouch et al., 2016; Carpio et al., 2016), but none of them actually investigated the potential exposure risk of reptiles towards pesticides, and they were either on a small scale, or did not target European reptiles, specifically. There has only been one study concerning the potential pesticide exposure risk of European reptile species by Wagner et al. (2015). In this study, the authors calculated the potential exposure risk to pesticides for different species by using different life history traits, physiology and presence/absence data of species. However, Wagner et al. (2015) only covered those species listed under Annex II of the Habitats Directive within their "Special Areas of Conservation" (SACs) (EU, 1992). Starting from this point, we decided to conduct a risk evaluation for as many reptile species as possible within their entire distribution range in the EU.

The goal of the present study was (1) to detect which taxa do generally occur within agricultural areas with regular pesticide applications and (2) to create an exposure index for all considered taxa by further taking into consideration life history and physiological traits of the species. We then (3) compared species occurring within the different pesticide admission zones within the EU (Regulation (EC) No 1107/2009) and evaluated, whether there are differences in pesticide exposure risk for each admission zone.

Methods

We proceeded using the method already applied by Wagner et al. (2015), albeit with slight modifications.

Identification of species occurring in agricultural areas

In this evaluation, we considered the risk of pesticide exposure of a species based on its regular presence or absence within agricultural areas. Contrary to Wagner et al. (2015), we only used literature data to determine species' occurrence within agricultural areas with regular pesticide applications (ARAs), since no pseudo-absence points for logistic regression analyses could be created at scale of the species' entire European range (for the creation of absence points in conservation areas, see Wagner et al. 2015). Data for species absence (as within SACs) was not available at the European scale. Thus, absence points within defined areas where species are known to be absent could not be generated in order to calculate logistic regressions using presence/absence of a species as predictor variable for general occurrence.

A regular occurrence within cultivated landscapes was only expected if evidence was found, i.e. multiple reports (≥ 3) attesting the presence of individuals in agricultural landscapes, known habitat preferences of a species or visual confirmation of species in the field. In case of enough evidence of a regular presence within ARAs (i.e. when at least one of the criteria was met), 1 Risk Point (RP) was awarded (otherwise 0 RP).

Species' physiology

This evaluation considered physiological factors of a species, which should increase the potential pesticide uptake. Additionally to snout-to-vent-length (SVL), which was also

evaluated by Wagner et al. (2015), we included average body mass (BM) in this evaluation. Data was retrieved from literature, and a classification scheme was established through the histogram function using the software R (R Developmental Core Team, Vienna). According to this method, SVL was classified into eight classes (0-10cm, 10-20cm, 20-40cm, 40-60cm, 60-80cm, 80-100cm, 100-120cm, 120-140cm), while BM was classified into eleven classes (0-10g, 10-20g, 20-40g, 40-60, 60-80g, 80-100g, 100-200g, 200-300g, 300-400g, 400-500g, >500g). The lower the BM and SVL, the higher the risk class a species was assigned to, as species with a small body size tend to exhibit a greater increase in dietary exposure when compared to larger species (Ellgehausen et al., 1980). Likewise, a small body size comes with a greater surface area, which can promote a higher dermal uptake of pesticides (Weir et al., 2010). 8 RP could subsequently be scored for SVL and 11 for BM. A total of 19 RP could thus be scored for physiology.

Species' life-history

This evaluation referred to life-history traits that may make populations of a species more or less susceptible to suffer from negative effects of pesticide exposure (mean number of clutches per year and mean clutch size). Time to reach sexual maturity – as used in Wagner et al. (2015) – could not be taken into account due to a great lack of data.

Species with a lower offspring and low clutch frequency (K-strategists) will probably suffer more from effects on individuals than r-strategists (Reznick et al., 2002). For instance, exposure concentrations for species with a low clutch frequency should be higher than for those with multiple clutches (Hopkins, 2006). A lower number of descendants will probably also lead to a decreased neonate survival, which could in turn cause decreasing population sizes (Guillette et al., 1994). The classification of clutch size followed the same pattern as for

physiology, using the histogram function in R. Clutch size was then classified into 7 classes: 1-3, 3-6, 6-9, 9-12, 12-15, 15-18, >18 eggs/descendants per clutch. For the amount of clutches per year, the actual number of clutches was used (1, 2, 3, 5, 6; none of the considered species laid 4 clutches in a year). 7 and 5 RP could be scored for clutch size and amount of clutches per year respectively. A total of 12 RP could thus be scored for life-history. Data on home ranges for different species could not be considered, as reliable information is lacking for a great majority of them.

Calculation of an Exposure Risk Index (ERI) and Exposure Risk Factor (ERF)

A final ’Exposure Risk Factor‘(ERF), which results from the combination of the proportion of ARAs within a species European distribution ranges and an ’Exposure Risk Index‘ (ERI, defined by the species’ scored RPs), was created for each taxon. This ERF reflects species’ potential pesticide exposure risk according to habitat exposure, physiology and life history, as well as the proportion of agricultural area within its European distribution. Species could score a different amount of RPs for each of these three evaluations. In order to equally weight all three of them, RP scores were summed relative to the maximum possible score for the respective evaluation. Thus, RP scores in all evaluations were converted to a 0-10 scale, so that each evaluation factor had the same impact on the final ERF.

Based on these evaluations, a species could score a maximum amount of 32 RP. Taking the weighted measures into consideration, this resulted in a maximum of 30 RP (as there are three evaluations with a maximum score of 10 RP each). The ERF was then calculated using a modified formula from Wagner et al. (2015) under which a species habitat can score 0 to 1 points:

$$ERF = ERI \times \frac{ARA}{30} \times 100$$

ERI = Sum of RP scored throughout all risk evaluations for the evaluated species.

ARA = Degree of overlap (%) between species ranges and agricultural areas with regular pesticide applications.

30 = Total amount of scorable RPs (after weighting).

ARA was calculated using the known European distribution range of species within the EU using ArcMap 10 (Esri®) and the latest version of the European CORINE (Coordination of Information on the Environment) land cover data (http://www.eea.europa.eu/).

Statistical analysis

Statistical analyses were conducted using the R software (R Developmental Core Team, Vienna). Assumptions of homogeneity of variance and normality were examined. Whenever these assumptions were not violated, parametric tests (ANOVA) were used to determine significant differences between ERI and ERF values among reptile orders and suborders, as well as IUCN red list conservation statuses and RPs scored between evaluation factors. In case data failed to meet these assumptions, Kruskal-Wallis tests were used in order to test for significant differences. Whenever significant differences could be observed between tested groups, Mann-Whitney U tests were performed to identify them. In order to test whether different pesticide admission zones display a higher amount of species occurring within ARAs, a Chi^2 test was used, as only the total number of species occurring in different land use types could be compared.

Results and discussion

Out of the 141 reptile species occurring within the EU (Cox and Temple, 2009), there was sufficient data available to evaluate the exposure risk of 102 species (72.34%).

Impact of physiology and life history on exposure risk

For both evaluations regarding species' physiology and life history, the majority of species were grouped into high risk classes (Table 1). This is the result of the relatively small size of most considered species, as a smaller body size will cause a greater exposure through food uptake (Weir et al., 2010). Similarly, a small body size comes with a greater surface area, which can promote a higher dermal uptake of pesticides (Ellgehausen et al., 1980). Finally, populations of species with fewer offspring, which additionally need more time to reach sexual maturity (K-strategists), are supposed to be more threatened by effects at the individual level compared to r-strategists (Guillette et al., 1994; Reznick et al., 2002).

Table 1: Number of species in different risk classes for evaluation factors concerning physiology and life history. Abbreviations: SVL – snout-to-vent length; BM – body mass.

SVL		**BM**		**Clutch Size**		**N° of Clutches/Year**	
Risk Class	**Species**	**Risk Class**	**Species**	**Risk Class**	**Species**	**Risk Class**	**Species**
1 (0-10cm)	1	1 (0-10g)	10	1 (1-3 eggs)	1	1 (1 clutch)	1
2 (10-20cm)	3	2 (10-20g)	1	2 (3-6 eggs)	3	2 (2 clutches)	1
3 (20-40cm)	5	3 (20-40g)	5	3 (6-9 eggs)	8	3 (3 clutches)	2
4 (40-60cm)	5	4 (40-60g)	5	4 (9-12 eggs)	10	4 (5 clutches)	16
5 (6-80cm)	11	5 (60-80g)	5	5 (12-15 eggs)	25	5 (6 clutches)	82
6 (8-100cm)	9	6 (80-100g)	4	6 (15-18 eggs)	27		
7 (100-120cm)	21	7 (100-200g)	4	7 (>18 eggs)	28		
8 (120-140cm)	47	8 (200-300g)	6				
		9 (300-400g)	8				
		10 (400-500g)	7				
		11 (>500g)	47				

When comparing the influence of physiology and life history on the ERF, life history seems to have a higher impact on the potential risk factor of a species. Here, no species scored an

amount of RPs < 5, and the average number of RP scores were significantly higher than for physiology (one-way ANOVA, $F_{1,202} = 6.20$, $p < 0.05$).

Exposure risk index (ERI) and exposure risk factor (ERF)

The average ERI for all species was 20.15 ± 5.52 out of a maximum of 30 obtainable RPs for a single species. The highest scores were achieved by *Algyroides fitzingeri, Chalcides bedriagai, Hemidactylus turcicus* and *Mediodactylus kotschyi* (30 RPs each). The lowest scores were obtained by *Dolichophis jugularis, Elaphe quatuorlineata, Elaphe sauromates* and *Malpolon insignitus* (< 10 RPs each).

A total of 44 species exceeded the average ERF of 0.23 ± 0.15 and can thus be considered to have an above average pesticide exposure risk. Out of these, the Spanish pond turtle (*Mauremys leprosa*) and the Greek tortoise (*Testudo graeca*) are listed as Vulnerable (VU) by the IUCN Red List of Threatened Species. Eight species are considered Near Threatened (NT), while 33 are listed as Least Concern (LC).

For the Sicilian pond turtle (*Emys trinacris*) and the Italian Aesculapian snake (*Zamenis lineatus*), data on conservation status is Data Deficient (DD) (Table 2). Overall, ERF values of Critically Endangered and Endangered species were significantly lower than for those classified as Near Threatened and Least Concern (Mann-Whitney U Test, $p < 0.05$). This can be explained by the narrow and isolated distribution ranges – often in remote areas where almost no agriculture is practiced – of the majority of Endangered and Critically Endangered species, such as the El Hierro giant lizard (*Gallotia simonyi*), which is an endemic species of the Canaries and only survives in small populations (Salvador, 2014).

A similar example can be observed with the Iberian rock lizard (*Iberolacerta monticola*) and Martinez-Ricas rock lizard (*Iberolacerta cyreni*), which are restricted to the Pyrenees and do not come into contact with agriculture (Molina-Borja and Rodríguez-Domínguez 1998, Martín, 2009a,b). At the same time, no significant differences could be observed between the studied reptile groups (testudines and squamata, subdivided in lacertilia and serpentes; Kruskal-Wallis-Test $\chi^2 = 5.32$, $df = 2$, $p > 0.05$) nor between families (Kruskal-Wallis-Test, lacertilia: $\chi^2 = 2.38$, $df = 4$, $p > 0.05$; serpentes: $\chi^2 = 8.16$, $df = 3$, $p > 0.05$ and testudines: $\chi^2 = 1.35$, $df = 2$, $p > 0.05$) based on ERF scores.

Table 2: Conservation status, proportion (%) of area with regular pesticide applications (ARA) within the European range, Exposure Risk Factor (ERF) and Exposure Risk Index (ERI) values of all species that surpassed the average ERF. Abbreviations for IUCN conservation status : CR – Critically Endangered, EN – Endangered, VU – Vulnerable, NT – Near Threatened, LC – Least Concern.

Species	**Common name**	**Conservation status**	**ERI**	**ERF**	**ARA (%)**
Gallotia stehlini	Gran Canaria giant lizard	LC	23.29	0.66	85
Mediodactylus kotschyi	Kotschy's gecko	LC	30	0.58	58
Testudo graeca	Greek tortoise	VU	22.54	0.56	74
Chalcides bedriagai	Bedriaga's skink	NT	30	0.52	52
Hemidactylus turcicus	Mediterranean house gecko	LC	30	0.52	52
Podarcis siculus	Italian wall lizard	LC	26.67	0.51	58
Coronella austriaca	Smooth snake	LC	24.34	0.49	60
Ablepharus kitaibelii	European copper skink	LC	20	0.47	71
Chalcides ocellatus	Ocellated skink	NT	26.75	0.47	53
Eremias arguta	Steppe-runner	NT	18.64	0.47	75
Anguis fragilis	Slow worm	LC	27.28	0.45	49
Tarentola mauritanica	Moorish wall gecko	LC	29.17	0.45	46
Teira dugesii	Madeira wall lizard	LC	29.17	0.45	46
Chalcides chalcides	Three-toed skink	LC	17.81	0.44	74
Chalcides striatus	Western three-toed skink	LC	18.64	0.43	69
Podarcis muralis	Common wall lizard	LC	28.33	0.43	46
Emys orbicularis	European pond turtle	NT	23.60	0.41	52
Psammodromus hispanicus	Spanish psammodromus	LC	29.17	0.41	42
Zootoca vivipara	Common lizard	LC	17.50	0.41	70
Typhlops vermicularis	European blind snake	LC	28.11	0.4	43
Lacerta agilis	Sand lizard	LC	27.50	0.39	43
Testudo hermanni	Hermann's tortoise	NT	21.71	0.38	52
Lacerta bilineata	Western green lizard	LC	25.90	0.37	44

Malpolon monspessulanus	Montpellier snake	LC	20.44	0.37	55
Podarcis hispanicus	Iberian wall lizard	LC	29.17	0.37	38
Chamaeleo chamaeleon	Common chameleon	LC	22.89	0.36	47
Darevskia praticola	Meadow lizard	NT	19.17	0.36	56
Gallotia atlantica	Atlantic lizard	LC	19.47	0.35	54
Lacerta viridis	European green lizard	LC	15.92	0.34	65
Macroprotodon cucullatus	False smooth snake	LC	28.42	0.33	35
Dolichopis caspius	Caspian whip snake	LC	19.80	0.31	48
Podarcis vaucheri	Andalusian wall lizard	LC	20	0.31	47
Gallotia galloti	Tenerife lizard	LC	18.11	0.3	49
Hemorrhois hippocrepis	Horseshoe whip snake	LC	22.54	0.3	40
Mauremys leprosa	Spanish pond turtle	VU	21.71	0.3	41
Podarcis pityusensis	Ibiza wall lizard	NT	20	0.3	45
Algyroides fitzingeri	Pygmy keeled lizard	LC	30	0.29	29
Timon lepidus	Ocellated lizard	NT	21.62	0.29	40
Acanthodactylus erythrurus	Spiny-footed lizard	LC	19.17	0.28	44
Emys trinacris	Sicilian pond turtle	DD	11.93	0.26	65
Podarcis bocagei	Bocage's wall lizard	LC	28.33	0.26	28
Telescopus fallax	European cat snake	LC	26.23	0.26	30
Platyceps najadum	Dahl's whip snake	LC	16.01	0.24	44
Zamenis situla	European ratsnake	LC	23.38	0.24	31

When comparing ERI values, however, there are significant differences between snakes and lizards (Mann-Whitney U Test, $p < 0.05$). This is explained by the higher amount of RPs the latter group scored for physiology and life history, as well as known occurrence within ARAs. Snakes on the other hand show more favorable physiological and life history traits. Concretely, gekkonids and lacertids display higher ERI scores than colubrids (Mann-Whitney U Test, $p < 0.05$ for gekkonids, $p < 0.01$ for lacertids), while viperids display higher scores than anguids (Mann-Whitney U Test, $p < 0.05$), gekkonids (Mann-Whitney U Test, $p < 0.01$), lacertids (Mann-Whitney U Test, $p < 0.01$) and scincids (Mann-Whitney U Test, $p < 0.05$). Accordingly, while no differences in exposure probability can be observed in their European distribution ranges, lizard populations are suggested to have an overall higher sensitivity toward pesticide exposure than snakes. The lack of differences between ERFs can be explained by the generally higher proportions of ARAs within snakes distribution ranges (see

supplementary material for further information), but lower RP scores for physiology and life history.

All threatened species which exceeded the average ERF coincided in their evaluated life-history characteristics. The amount of clutches laid per year, as well as the number of eggs they may lay per clutch is low. For instance, offspring of species with lower clutch frequencies may have a higher probability of suffering increased exposure concentrations as opposed to those with multiple clutches, especially if the oviposition overlaps with pesticide applications (Hopkins, 2005). A lower number of offspring can lead to a decreased survival rate of neonates, which could in turn cause a decreasing population size (Guillette et al., 1994). At the same time, the majority of species with an above average ERF showed a high proportion of ARAs within their European distribution range (50% on average).

The results of our present study generally correspond with those of Wagner et al. (2015) concerning the risk evaluation of pesticide use to protected European reptile species within their conservation areas. Wagner et al. (2015) only contemplated SACs established in the EU. Agricultural land use within these areas is legal, although it is bound to defined conditions and is more restricted (http://ec.europa.eu/; EU, 1992). It would thus not have been surprising if exposure risk from both studies would have diverged more significantly when the entire European distribution range of a species is considered. In our current study, *Lacerta schreiberi* and *Testudo marginata* scored lower ERF values than in the work of Wagner et al., while *Podarcis pityusensis* and *Gallotia galloti* scored higher.

Altogether, 30% of all reptile species occurring in the EU (Cox and Temple, 2009) display an above average pesticide exposure risk. This does not only affect non threatened species, but also some Vulnerable species. If not considered, ignoring exposure of reptiles towards

pesticides could lead to increasing regional species loss, and may possibly increase the number of threatened species in the long run.

Seeing that especially lizards scored the highest ERI values, and given that squamates are particularly underrepresented in ecotoxicological studies (Amaral et al., 2012a; Sparling et al., 2010; Weir et al., 2010), this indicates the urgent need to act and integrate reptiles into pesticide risk assessments for future admission procedures, as well as into conservation practice.

Differences between Mutual Recognition Zones

Differences regarding the occurrence of species within ARAs can be observed between pesticide admission zones: The highest number of species occurring in ARAs can be observed in the southern zone (Figure 1, χ^2-Test $_{(2,\ N\ =\ 141)}$ = 8.01, $p < 0.05$), where the majority of species occurs within arable land (about half of them in cereal plantations). Here, the most occupied agricultural areas besides arable land are vineyards and olive groves. This can be explained by the higher amount of reptile species occurring in Mediterranean countries (Cox and Temple, 2009), and the subsequently higher number of potential species inhabiting agricultural areas. Simultaneously, some of these ARAs (i.e., citrus plantations and olive groves) are restricted to countries in southern admission zones, and southern countries are characterized by an overall greater area dedicated to agriculture (Eurostat, 2014). In the central admission zone, the most occupied ARAs are still arable land and vineyards, while the importance of orchards as exposing area drops drastically. The increasing prominence of vineyards as important contaminant habitats can be explained due to the ecology and habitat preferences of originally Mediterranean species, such as the common wall lizard (*Podarcis muralis*) or the western green lizard (*Lacerta bilineata*) for example. These species naturally

occur in a multitude of habitats in warm southern climates, but are bound to dry and rocky areas with low vegetation - which are often used for viniculture - in their northern distribution ranges (Böhme, 1981; Schulte, 2008). Incidentally, the highest amount of pesticides used by crop in the entire EU lays within “grape plantations”, with > 20 kg of active substance/ha (Eurostat, 2007). In the northern zone, a drastic decrease in occupied ARAs can be observed, which is mainly determined by the low number of reptile species occurring within these countries, and the lower population sizes, coupled with more restricted frequencies (Cox and Temple, 2009).

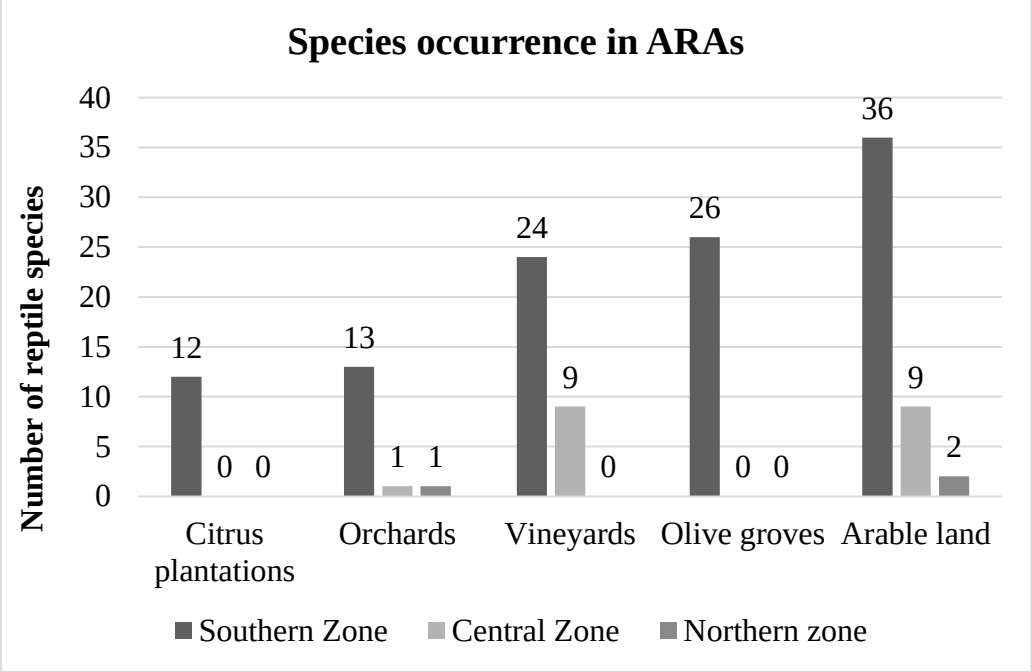

Figure 1: Number of reptile species with general occurrence within selected ARAs for each of the three Mutual Recognition Zones within the EU (Regulation (EC) No 1107/2209).

As species’ abundance in the southern admission zones is higher, and the incidence rates in ARAs seem to be higher than in the central zones, but especially the northern admission zones, the integration of pesticide assessments into conservation practice is of special importance for southern countries. Here, species diversity is higher, and populations are expected to suffer from pesticide exposure. Effectively protecting species in these countries

could thus contribute to biodiversity preservation in the entire EU. At the same time, the possibility that reptile species persisting in agricultural areas of southern countries might undergo more exposure events when compared to those in northern admission zones may be an additionally crucial factor, as pests may meet more favorable thriving conditions than in harsher northern climates. Multiple pesticide applications may be needed in order to successfully eradicate pests relative to the amount needed in a more unfavorable climate to them (Food and Agriculture Organization of the United Nations, 2008). Furthermore, pests may become more resistant to pesticides (Brattsten et al., 1986), which increases the number of applications and doses needed to cope with them. Coincidentally, the countries with the highest pesticide application rates originate mainly from the southern admission zone (e.g. France, Spain, Italy, Portugal) (Eurostat, 2007). This does not mean, of course, that protection measures in the northern and central zones should be disregarded.

Conclusions

Our results show that at least one third of the considered European reptile species display an increased pesticide exposure risk, and two threatened species (*Testudo gracea* and *Mauremys leprosa*) within the EU fall under this category. These results strongly indicate that pesticide risk assessments and exposure assessments need to be integrated into conservation practice in order to help improve conservation processes for reptiles and avoid further biodiversity loss.

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CHAPTER II

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

Valentin Mingo[1], Stefan Lötters[1] and Norman Wagner[1]

[1]Trier University, Department of Biogeography, Universitätsring 15, 54296 Trier, Germany.

Highlights

- Buccal swabs could become a reliable method to detect pesticide exposure in reptiles.
- Biomarker data from wild lizards matches results obtained in laboratory studies.
- Fungicide formulations induced oxidative stress in exposed individuals.
- Reptiles suffer from pesticide uptake, and need to be integrated in risk assessments.

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.

Capsule abstract

Examined pesticides caused oxidative stress in exposed individuals from the wild. Buccal swabs could become a reliable tool to detect pesticide exposure in reptiles.

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 Hosek 2016, http://www.reptile-database.org; accessed 25.05.2016). As a result, squamates are

especially 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; Amaral et al. 2012b; Amaral et al. 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.

Materials and Methods

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 to 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.

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

We selected *Podarcis muralis* as study species for pesticide 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. over-sprayed arthropods.

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.

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).

Enzymatic assays

Frozen buccal swabs were thawed on ice and subsequently homogenized with a Mini-Beadbeater-24 homogenizer (Biospec®). Lysis buffer consisted of 25mM Tris-HCl and 0.1% Triton X-100. Samples were homogenized for 45 sec 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 µmol/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 µmol/mg^{-1} protein/min, using a molar extinction coefficient of 1.36×10^4 $M^{-1}cm^{-1}$. 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).

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-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).

Results

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

GST activity

Figure 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, $p < 0.05$).

Figure 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, $x^2 = 12$, $df = 1$, $p > 0.05$).

GR activity

Figure 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, $p < 0.05$) (Figure 2 c, e, g).

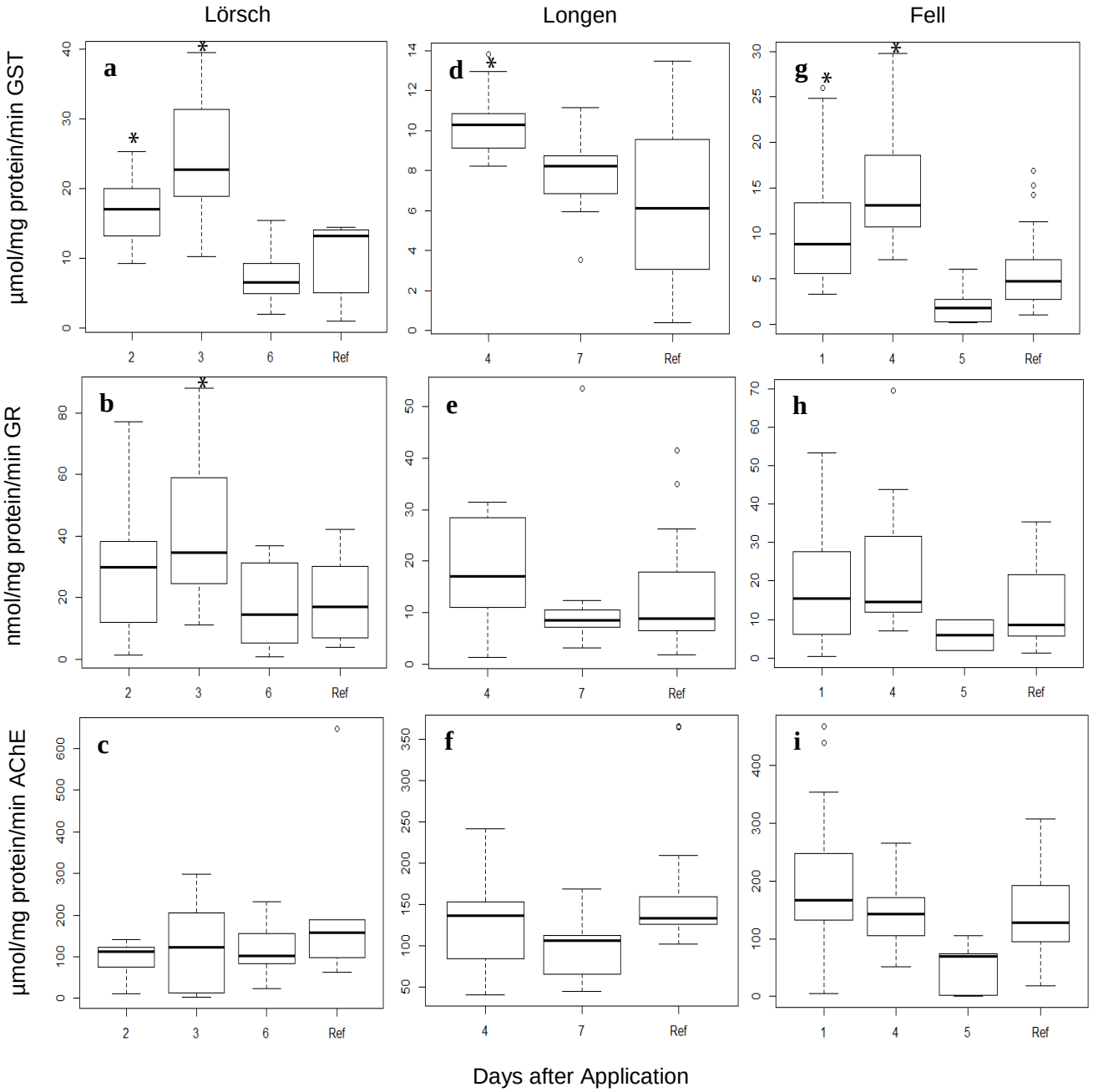

Figure 1: GST, GR and AChE activity rates for studied individuals exposed to fungicides along the three sampling sites. GST activity rates are depicted in sections **a**, **d** and **g**. GR activity rates are represented in sections **b**, **e** and **h**, while AChE is depicted in sections **c**, **f** 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, $p < 0.05$).

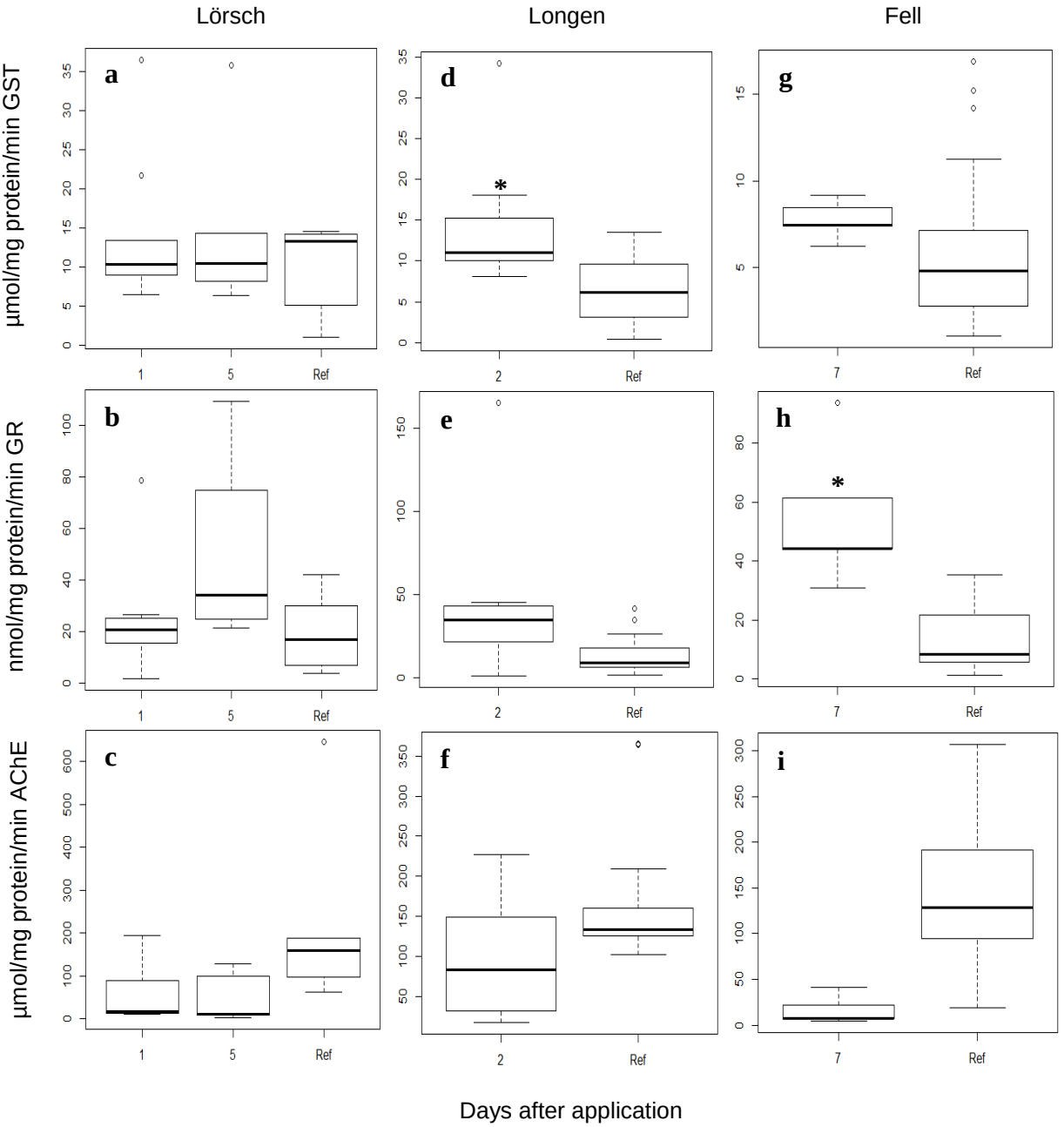

Figure 2: GST, GR and AChE activity rates for studied individuals exposed to the herbicide Touchdown® along the three sampling sites. GST activity rates are depicted in sections **a**, **d** and **g**. GR activity rates are represented in sections **b**, **e** and **h**, while AChE is depicted in sections **c**, **f** and **i**. Abbreviations as in Figure 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, $p < 0.05$).

AChE activity

Figure 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, $x^2 = 2$, $df = 1$, $p > 0.05$) (Figure 2 c, f, i).

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 Figure 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, $p > 0.05$, $df = 174$, $r = 0.0023$; Figure 3b). Between GR and AChE, a negative correlation was identified over all samples (Pearson correlation, $p < 0.05$, $df = 148$, $r = -0.16$; Figure 3c).

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 ($p < 0.05$, $rho = 0.61$; Figure 4a), while AChE activity rates did not ($p > 0.05$, $rho = 0.22$; Figure 4b). For GR, again a positive correlation could be shown ($p < 0.05$, $rho = 0.46$; Figure 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 ($p < 0.05$, $r^2 = 0.52$).

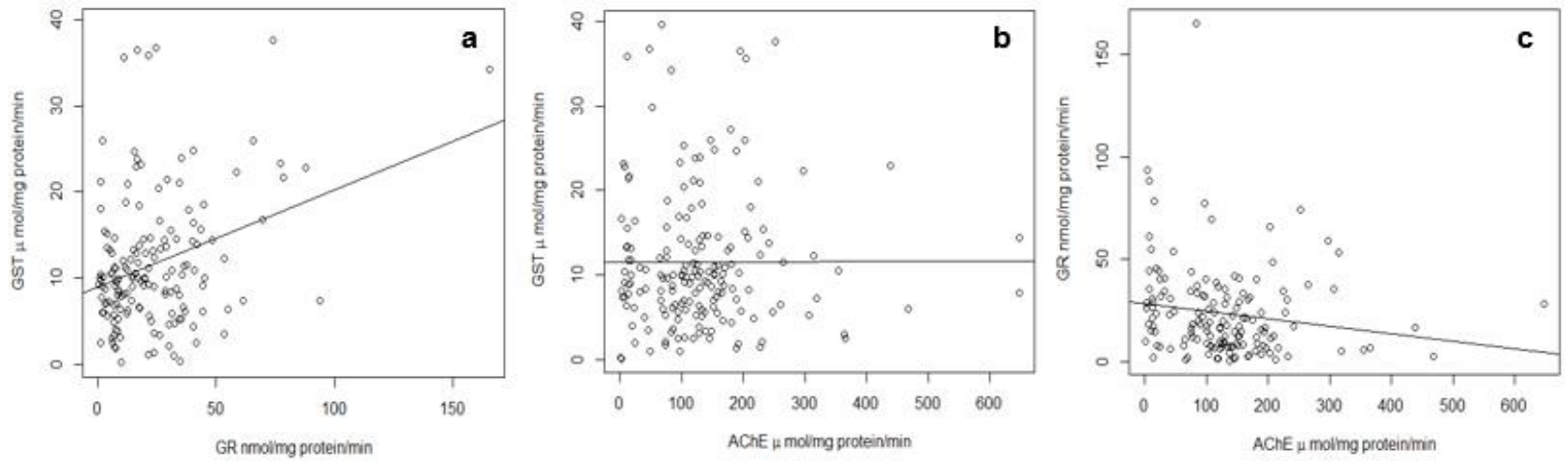

Figure 3: Scatterplot showing correlations between **a)** GST and GR activity rates ($p < 0.001$, $df = 155$, $r = 0.32$), **b)** GST and AChE activity rates ($p > 0.05$, $df = 174$, $r = 0.0023$) and **c)** GR and AChE activity rates ($p < 0.05$, $df = 148$, $r = -0.16$) for all saliva samples.

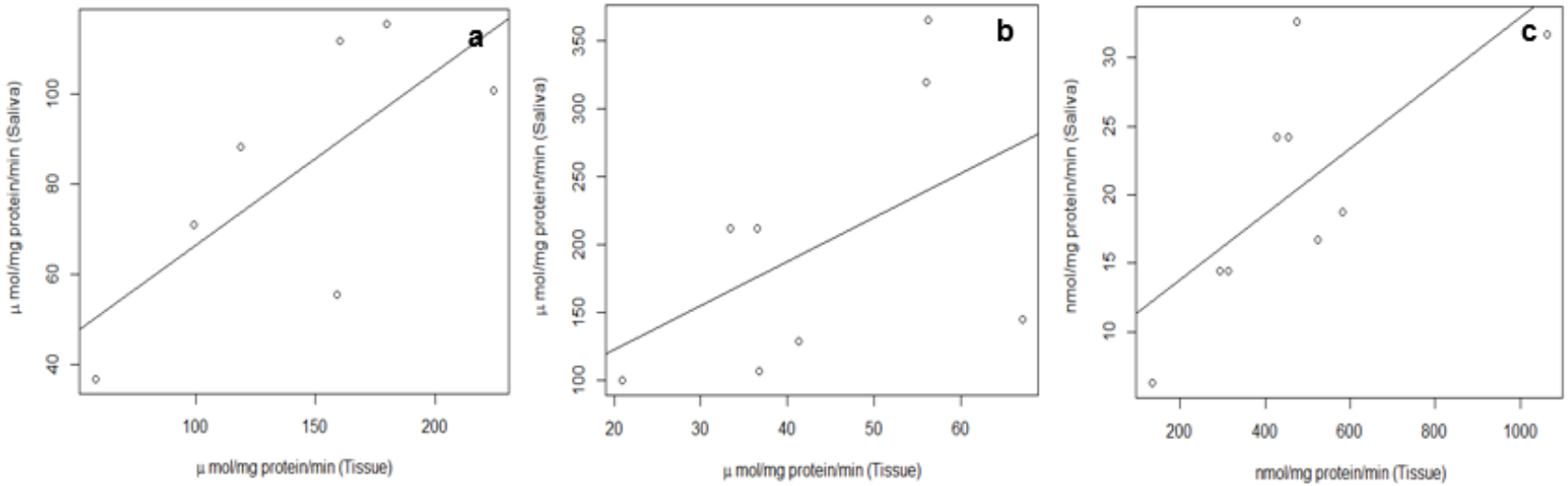

Figure 4: Scatterplots showing activity in tissue and saliva samples of the studied individuals for **a)** GST ($p < 0.05$, *rho* = 0.61), **b)** AChE ($p > 0.05$, *rho* = 0.22) and **c)** GR ($p < 0.05$, *rho* = 0.46).

Discussion

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 within a 1km buffer surrounding the sampling site.

For Longen and Fell, agricultural land use (vineyards again) only amounted to 40% and 10% of the area within 1km 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 to 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, Figure 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 (Figure 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.

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.

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.

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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Validating buccal swabbing as a minimal-invasive method to detect pesticide exposure in reptiles

Valentin Mingo[1], Christoph Leeb[2], Ann-Katrin Fahl[2], Carsten Brühl[2], Stefan Lötters[1] and Norman Wagner[1]

[1]Trier University, Department of Biogeography, Universitätsring 15, 54296 Trier, Germany

[2]University Koblenz-Landau, Institute for Environmental Sciences, Forststraße 7, 76829 Landau, Germany

Highlights

- Buccal swabs are a reliable method to detect effects of pesticide exposure
- Separate effects of dermal and oral exposure pathways can be observed
- Only a fungicide mix had a significant effect on locomotor performance of lizards
- Exposed lizards showed a longer basking activity than those of the control group
- Food consumption was not affected after exposure to any pesticide

Abstract

The use of enzymatic biomarkers constitutes a widely used approach in ecotoxicology. However, standard sampling procedures are invasive, requiring tissue, organ or blood extraction. This leads to concerns regarding conservation practice, animal welfare and restrictions in study design. New techniques are needed to avoid these problems, but still generate reliable data. In previous work, we proposed the use of buccal swabs as a minimally invasive method to assess effects of pesticide exposure in reptiles, a vertebrate group threatened by worldwide biodiversity loss, for which knowledge regarding effects of pesticide exposure is still very scarce. Here, we validate our previous field-based data under laboratory conditions. We are able to establish a clear link between pesticide exposure and enzymatic biomarker response. Common wall lizards (*Podarcis muralis*) were divided into three treatment groups (control, dermal and oral), exposed to different pesticide formulations (a mixture consisting of the fungicide formulations Enervin® and Vivando®, the single fungicide formulation Vivando® and the herbicide Roundup UltraMax®) and buccal swabs were taken as in previous field studies. To the best of our knowledge, this is the first work dealing with exposure of squamate reptiles to pesticide mixtures. Aside from enzymatic activity, additional endpoints, i.e. locomotor performance, basking behaviour and food consumption were compared. Results regarding enzymatic activity matched with previous field data, and a clear cause-effect relationship between pesticide exposure and enzymatic activities could be observed. Regarding locomotor performance and basking activity, the strongest effect was observed after exposure to the fungicide mixture (Enervin® + Vivando®). Our results strongly advocate that buccal swabbing is a reliable minimal invasive method to generate samples for detecting effects of pesticide exposure in reptiles. Due to its easy handling, we believe it will provide new opportunities concerning study designs.

Capsule abstract

In the laboratory, we confirmed the suitability of buccal swabbing as a reliable, minimal-invasive method to detect effects of pesticide exposure in Common wall lizards (*Podarcis muralis*) using enzymatic biomarker assays. Further, exposure to pesticides resulted in changes regarding behavior and locomotor performance of individuals.

Introduction

As found in various plant and animal groups, reptiles are suffering from global population declines (Gibbons et al. 2000, Todd et al. 2010, Weir et al. 2010). These dramatic declines go as far as threatening 15-36% of the worldwide reptile diversity (Böhm et al. 2013). Within the European Union (EU), 20% of all reptile species are considered 'threatened' by the IUCN Red List of Threatened Species. Independently of it, 41.7% of all European reptile species are estimated to be in decline by Cox and Temple (2009). The main causes for declines have been identified, among others, as habitat loss and degradation, coupled with environmental pollution. The latter being in many cases caused by pesticide use (Gibbons et al. 2000, Todd et al. 2010, Sparling et al. 2010). While being an important factor contributing to diversity loss, data regarding effects of pesticide exposure in reptiles is still scarce. First, because studies concerning this topic are largely lacking (Hopkins 2000, Campbell and Campbell 2002). Second, because in studies that have actually addressed this topic, the order of squamata (lizards and snakes) has been widely neglected (Campbell and Campbell 2002). This is rather unbalanced as almost 95% of the worldwide reptile diversity resides within this order (Uetz and Hošek 2017). This makes it clear that our knowledge regarding the topic is very limited, and leaves a large data gap which needs to be filled in order to assess the actual impact pesticide applications may have on reptiles.

The use of enzymatic biomarkers has become a powerful tool whilst studying these effects. It plays an important role in the field of ecotoxicology, as it allows to detect exposure events and their specific effects at the sub-individual level. All in all, this is a field that has been continuously improved and, as of now, a multitude of different enzymatic markers have been established to detect a multitude of effects in different taxa (Sparling et al. 2010, Lajmanovich et al. 2011, Mecdad et al. 2011, Amaral et al. 2012b, Carvalho et al. 2013, Murussi et al. 2014, Kori et al. 2016). Conversely, this progress has not been translated into the development of less invasive sampling methods. In fact, one of the main controversies regarding this technique in wildlife ecotoxicology is that it requires tissue, organs or blood (Fossi 1994, Lajmanovich et al. 2011, Amaral et al. 2012b, Kori et al. 2016). While organ extraction commonly means death, blood sampling is comparatively harmless, but mainly in larger animals. For smaller animals however, this is rather straining. In the case of small reptiles, for instance, blood sampling is normally achieved by cardiac puncture which is highly risky, too (Aldridge et al. 1990, Dodd 2016). Apart from ethical aspects, this entails serious concerns regarding permissions and practicability, as research studies are often performed in a conservation framework. In the EU, in particular, legislation regarding protection of animals used for scientific purposes is strict, even more so for strictly protected species listed under Annex IV of the habitats directive (European Council 1992, European Parliament and Council, 2010), which is the case for many European reptile species.

As a consequence, the use of invasive methods poses a remarkable dilemma for strictly protected and endangered species. The implementation of less invasive techniques is therefore an important goal in order to be able to better assess effects of pesticide exposure. Regarding this topic, the use of saliva has been considered a potential matrix for enzymatic analyses, as it represents a simple and readily obtainable fluid in which biomarker exposure can be

assessed (Kori et al. 2016). For instance, Henn et al. (2006) proposed the use of saliva as non-invasive method in human pesticide biomonitoring, using Acetylcholinesterase (AChE) as biomarker. Wang et al. (2015) reviewed the use of saliva as a medium to analyze metabolomics of biomarkers of oxidative stress in humans and concluded that it indeed constitutes a promising area for biomarker discovery in a wide array of biomedical conditions. Regarding lizards, for instance, Schulte et al. (2011) have shown that buccal swabbing is a reliable method for DNA sampling. Based on these observations, we explored using saliva samples (via buccal swabbing) as a means to detect effects of pesticide exposure in lizards. During previous field studies (Mingo et al. 2017a,b) we assessed the practicability of this method by taking buccal swabs from wild lizard populations from different wine growing areas and measuring the effects of pesticide exposure on the enzymatic biomarkers Glutathion-S-Transferase (GST), Glutathion Reductase (GR) and Acetylcholinesterase (AChE). We were able to detect specific effects on enzymatic activity shortly after a pesticide application had taken place. However, field studies are difficult to standardize and the nigh impossibility to discern between synergies and/or antagonistic effects remains. Furthermore, whether additional field parameters may have influenced enzymatic activity is difficult to assess. Therefore, we saw that a standardized laboratory study was required to ascertain a direct cause-effect relationship between enzymatic activity and pesticide exposure.

To this end, Common wall lizards (Squamata: *Podarcis muralis*) were exposed to conventional field doses (FD) of three pesticides under standardized laboratory conditions. Individuals were exposed to: (1) a fungicide mixture consisting of Enervin® and Vivando®; (2) the single fungicide formulation Vivando® and (3) the widely used herbicide Roundup® UltraMax. Additional endpoints including behavioral parameters, body mass and locomotor

performance were recorded for all individuals and compared between treatment groups, as a means to detect further exposure effects.

Our main questions were:

- Are previous results of enzymatic biomarker assays using buccal swabs from contaminated wild lizard populations comparable to results from the present laboratory study?
- Is the method suitable to detect pesticide uptake through both dermal and oral exposure pathways, and do these exposure routes lead to different effects in lizards?
- Does exposure to test substances affect food consumption of lizards during the study?
- Does exposure lead to behavioral changes in thermoregulation or induce clinical signs regarding locomotion?

Materials and methods

Study species

The Common wall lizard (*Podarcis muralis*) was selected as model species, following Mingo et al. (2017a,b). Within its northern distribution range (Rhineland-Palatinate, Germany) its main habitat consists of steep slopes mainly exploited for viniculture (Schulte 2008). Therefore, it is a species known for its strong ties to agriculture, and regularly comes into contact with pesticides (see Mingo et al. 2017a,b). According to Eurostat (2007), 'grape plantations' display the highest amount of pesticides used by crop in the EU, with >20 kg of active substance/ha. 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

(Schulte 2008, Wagner et al. 2015, Mingo et al. 2017a,b). As a result, both oral (food items) and dermal (overspray) pesticide exposure within its habitat have to be taken into account.

A total of 30 Common wall lizard specimens (18 males and 12 females, 4 of which were juveniles) were retrieved from a population in the city of Mannheim (Germany). Lizards of this population belong to a genetic lineage originating from Italy (*Podarcis muralis nigriventris*), and were introduced during the 20th century (Schulte et al. 2012). Sampling permissions were granted by the Regierungspräsidium Karlsruhe.

Reptile housing

Specimens were housed individually in glass terraria with dimensions of 30 x 20 x 20 cm. Room temperature was 18–24 °C, with a relative humidity of 50–70% and a light-dark rhythm of 16:8 hours. All terraria were provided with relevant habitat components: hiding places, dig and climbing opportunities, as well as basking spots for thermoregulation. Water was provided *ad libitum*, for feeding see below. Prior to commencing the study, housing conditions were granted by veterinary authorities.

Studied pesticide formulations

Three widely used pesticide formulations were selected, which are commonly used in viniculture (Mingo et al. 2017a,b, BVL 2017). Two of them were the fungicide formulations Vivando® and Enervin®, given that the major part of pesticide applications in vineyards are constituted by this class of pesticides (Eurostat 2007, González-Rodríguez et al. 2009, Mingo et al. 2017a,b). Fungicides are usually applied in a pesticide mix of two or three formulations. For this reason, we tested a pesticide mixture of Enervin® and Vivando® (Table 1). Applied FD for Vivando® is very low (ten times lower than that of Enervin®) (Mingo et al.2017a,b). Thus, exposure to these kind of formulations can be expected to be minimal, compared to

other formulations, constituting a “worst case” scenario for effects detection. Therefore, in a second step, Vivando® was tested as a single formulation. This way, we aimed at verifying whether exposure to these ‘minimal’ application doses can be attested via enzymatic analyses using buccal swabs. As Enervin® by itself has to be considered a pesticide mix (its active ingredients (a.i.) being Initium® and Metiram®), the formulation was not tested separately as both active substances should be assessed individually in order to discern between effects. However, the goal of the study was not to provide specific data towards which pesticide formulations or a.i. may be more ecotoxicologically relevant. The third formulation tested was the glyphosate-based herbicide Roundup® UltraMax. While comparatively infrequent, herbicides are applied one to two times a year in order to control weeds (Mingo et al. 2017a,b). The tested concentrations of each formulation corresponded to normal FD, as used in viniculture (Mingo et al. 2017a,b), and were adjusted to the lizard’s housing dimensions (Table 1).

Uptake pathways

To discern not only between effects of pesticide formulations, but also exposure pathways, lizards were divided into three treatment groups: control, dermal and oral. Each group consisted of 10 individuals. Animals were assigned randomly to each treatment group, while maintaining equal sex ratios (6 males and 4 females).

Oral exposure

Before starting the exposure trials, animals were fasted for one week in order to ensure prey ingestion. Lizards of this group were fed contaminated prey items. These food items (domestic cricket, *Acheta domesticus*) were obtained from a commercial breeder and did not come into previous contact with any of the tested formulations. Before feeding, crickets were

over-sprayed with conventional FD, as used in viniculture, which were adjusted to the dimensions of cricket tanks (i.e. the same as those for lizards; Table 1). Prey items were over-sprayed once at the beginning of each trial (day 0) and offered to the lizards throughout a time period of 4 days (96h). Each lizard was provided with 5 crickets at day 0 in order to ensure sufficient food availability. During the following days of each trial (24h – 96h), crickets were resupplied to 3 individuals per lizard in order to guarantee sufficient food availability.

Dermal exposure

Lizards were directly over-sprayed with pesticide formulations, after all structure elements and hiding places, except for bare soil, had been removed from terraria. Concentrations of pesticide formulations were the same as the ones used to over-spray food items (Table 1). Exposure for each application only took place at the beginning of each trial (day 0), and was not renewed during the testing period of 96 h. Individuals of the dermal exposure group were fed exclusively uncontaminated crickets from the same commercial breeder.

Control group

Except for not being exposed to any pesticides during the trial, animals of the control group were treated equally to those of exposed groups and fed the same amount of uncontaminated crickets.

Table 1: Pesticide concentrations used to overspray food items and wall lizards, according to regular field doses (FD), adjusted to the housing conditions

Formulation	**FD kg, l/ha**	**Adjusted Concentration (600 cm^2)**
Enervin®	4	24 µg
Vivando®	0.4	2.4 µl
Mix (Enervin® + Vivando®)	4 + 0.4	24 µg + 2.4 µl
Roundup® UltraMax	4	24 µl

Trial duration

A time frame of 4 days (96 h) to detect effects of pesticide exposure on enzymatic activity was considered reasonable, as a similar time frame was examined during previous field studies (Mingo et al. 2017a,b). Due to animal welfare and conservation concerns and to reduce animal testing, 30 wall lizards were caught and tested. After each exposure trial, individuals were given a minimum of 10 days for recovery and were subsequently randomly reassigned to treatment groups, while keeping equal sex ratios. *In situ*, normalization of enzymatic activity in lizards was achieved after a maximum of six days (Mingo et al. 2017a,b). Ten days were thus considered an adequate time interval to exclude interferences between trials. All individuals were released at their sampling place in Mannheim at the end of the study.

Studied endpoints

Buccal swabbing, enzymatic biomarkers and assays

Saliva samples of individuals from each treatment group were retrieved daily using conventional cotton swabs in the manner of Mingo et al. (2017a). Individuals were swabbed prior to being exposed, at 0h, so that enzymatic activities measured in these samples can be considered as reference activities for each treatment group, respectively. Swabs were stored at -80°C until analyses started. The analyzed biomarkers were Glutathione-S-Transferase (GST), Glutathion Reductase (GR) and Acetylcholinesterase (AChE).

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 different contaminants, including pesticides in reptiles (e.g., Lajmanovich et al. 2011, Amaral et al.

2012b). It is considered a widely used and 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 studies concerning the exposure of different organisms to pesticides and other xenobiotic substances, and is considered a reliable biomarker to detect oxidative stress (Amaral et al. 2012b). AChE is a crucial enzyme that serves to terminate synaptic transmission, by catalyzing the breakdown of acetylcholine and other choline esters that function as neurotransmitters (Quinn 1987). It is the primary target of inhibition by organophosphorus compounds such as nerve agents and some pesticides (Quinn 1987, Tougu 2001). AChE is widely used to assess neurotoxic properties of pesticides on vertebrates (Gavric et al. 2015).

Enzymatic assays for the determination of GST, GR and AChE activity rates were performed as described in Mingo et al. (2017a).

Biometric parameters and clinical signs (food consumption, thermoregulation and locomotor performance)

All individuals were weighed at the beginning and end of each trial, and snout-to-vent-length (SVL) was recorded. These data were used as co-factors in statistical analyses. Furthermore, pesticide exposure could induce changes in body mass, as observed under natural conditions (Amaral et al. 2012b, Mingo et al. 2017b). Also, reptile species may develop avoidance behavior regarding contaminated food items (Yanes-Marichal et al. 2017). Feeding behavior was observed by counting the amount of consumed prey items per individual per day. There are known cases of “fever responses” of reptiles towards environmental contaminants

(Carpenter et al. 2016). Being poikilothermic vertebrates, this response allows reptiles to increase their metabolic rate in order to metabolize and eliminate xenobiotic substances faster (Talent 2005). Changes in thermoregulation behavior were monitored by observing the basking behavior of individuals. Additionally, temperature on lizard's backs was compared with that of their surroundings (within their terraria) using an infrared thermometer. Basking behavior was assessed by observing the amount of individuals basking within each treatment group during each day at 9 a.m., 1 p.m. and 5 p.m.

There are several reported cases of environmental contaminants affecting locomotor performance in reptiles (Hopkins and Winne 2006, DuRant et al. 2007, Amaral et al. 2012b). Impairments in locomotion can play a critical role in the survival of reptiles, influencing the ability of individuals to avoid predators, defend territories, mate or acquire food. In order to investigate whether any of the tested pesticide applications had an effect on locomotor performance, running and climbing performance of individuals was measured at 0h (before exposure), 24h, 48h, 72h and 96h after exposure. Before starting the trials, lizards were warmed to their optimal temperature (~32 °C; Schulte 2008) using an incubator (HerpNursery II® from Lucky Reptile), and exact temperature was recorded using an infrared thermometer. Lizards were hand-chased, causing a flee response, and trials were recorded using a DSLR-Camera (Nikon D500). Videos were analysed using the FrameShots software (EOF Productions). Running performance was measured racing the individuals through a 2 m long and 10 cm wide track made of compressed wood. Climbing performance was measured with the same equipment covered with a textile mesh and at an inclination of 65°. Locomotor performance was measured as meters per second (m/s) in both cases. Individuals were each raced two times and mean time to finish the track was used.

Statistical analyses

All analyses were conducted using the *R* software (R Developmental Core Team, Vienna). Assumptions of homogeneity of variances (Levene test) and normality (Kolmogorov-Smirnov test) were examined. In case data failed to meet these assumptions, transformations were performed to fit the data to normal distributions (Freedman et al. 2007).

Enzymatic activities were compared within exposure groups, using a repeated-measures-ANOVA, as alterations in enzymatic activity depend from the moment exposure took place. Changes in body mass were compared between treatment groups after each exposure trial using a one-way-ANOVA. Comparison of feeding behavior took place between exposure groups, using SVL and body mass of individuals as co-variates in an ANCOVA. Basking abundance was then compared using a Chi^2-test (Freedman et al. 2007).

Locomotor performance was compared between treatment groups (control, dermal, oral), but also within groups between trials (0h, 24h, 48h, 72h, 96h). For the analysis of within-group differences, repeated-measures-ANOVAs were employed in order to detect significant differences, as comparisons encompass the same individuals, but alterations in locomotor performance were expected to be influenced by treatment. In order to detect between-group differences, ANCOVAs were employed, using body mass, SVL, temperature and gender as co-variables (Freedman et al. 2007).

Results

Enzymatic biomarker activity – Fungicide mixture (Enervin® + Vivando®)

Enzymatic activities for the studied biomarkers are provided in Table 2. No significant differences were observed for any of them during any day after exposure in the control group (GST: repeated-measures-ANOVA, $df = 4$, $x^2 = 9.99$, $p > 0.05$ / GR: repeated-measures-

ANOVA, *df* = 4, x^2 = 23.441, p > 0.05 / AChE: repeated-measures-ANOVA, *df* = 4, x^2 = 16.42, p > 0.05). Within the dermal exposure group, a significant increase in GST activity was found between days 0 and 2 after exposure (repeated-measures-ANOVA, *df* = 4, x^2 = 11.006, p < 0.05; Bonferroni post-hoc test day 2, p < 0.05) and days 0 and 2 after exposure for GR (repeated-measures-ANOVA, *df* = 4, x^2 = 5.6, p < 0.05; Bonferroni post-hoc test day 2, p < 0.05). No effects were noted concerning AChE activity. Regarding the orally exposed group, GST activity was significantly increased during days 1, 2 and 4 after exposure (repeated-measures-ANOVA, *df* = 4, x^2 = 28.5, p < 0.05; Bonferroni post-hoc test, p < 0.05). An increasing in GR activity was recognized during day 2 after exposure (repeated-measures-ANOVA, *df* = 4, x^2 = 5.538, p < 0.05; Bonferroni post-hoc test, p < 0.05) . Again, no effects on AChE activity could be attested.

Table 2: Enzymatic activity rates (µmol/mg protein/min^{-1}) for the studied biomarkers (GST, GR, AChE), for all treatment groups after exposure to the fungicide mix (Enervin® + Vivando®).

Biomarker	GST			GR		
	Control	Dermal	Oral	Control	Dermal	Oral
Day 0	198.05 ± 30.89	215.54 ± 63.70	193.14 ± 39.81	22.4 ± 6.1	16 ± 5.6	20.7 ± 9.4
Day 1	262.41 ± 98.72	216.46 ± 97.92	248.6 ± 89.52	22.2 ± 5.8	14 ± 7	22.4 ± 10.2
Day 2	207.64 ± 116.92	272.95 ± 117.32	298.78 ± 126.49	18 ± 3.4	22.1 ± 9.8	27.3 ± 15.1
Day 3	253.45 ± 97.11	192.8 ± 48.76	226.34 ± 67.13	14 ± 7.8	21.4 ± 8.3	21.9 ± 11.1
Day 4	212.94 ± 83.54	186.53 ± 83.44	172.08 ± 49.77	17.6 ± 12.9	14.8 ± 6.4	20.6 ± 5.6

Biomarker	AChE		
	Control	Dermal	Oral
Day 0	0.58 ± 0.22	0.53 ± 0.26	0.46 ± 0.13
Day 1	0.51 ± 0.22	0.47 ± 0.21	0.50 ± 0.14
Day 2	0.47 ± 0.26	0.51 ± 0.11	0.48 ± 0.19
Day 3	0.80 ± 0.6	0.41 ± 0.07	0.56 ± 0.22
Day 4	0.60 ± 0.23	0.42 ± 0.13	0.45 ± 0.09

Enzymatic biomarker activity – single fungicide formulation (Vivando®)

Enzymatic activities for all studied biomarkers after exposure to Vivando® can be retrieved from Table 3. No differences in activity rates were found for individuals of the control group (GST: repeated-measures-ANOVA, *df* = 4, x^2 = 9.07, p > 0.05 / GR: repeated-measures-

ANOVA, *df* = 4, x^2 = 13.98, p > 0.05 / AChE: repeated-measures-ANOVA, *df* = 4, x^2 = 17.44, p > 0.05). Neither significant changes in enzymatic activity could be detected for individuals of the oral (GST: repeated-measures-ANOVA, *df* = 4, x^2 = 21.53, p > 0.05 / GR: repeated-measures-ANOVA, *df* = 4, x^2 = 10.50, p > 0.05 / AChE: repeated-measures-ANOVA, *df* = 4, x^2 = 5.39, p > 0.05) nor dermal group (GST: repeated-measures-ANOVA, *df* = 4, x^2 = 9.53, p > 0.05 / GR: repeated-measures-ANOVA, *df* = 4, x^2 = 20.27, p > 0.05 / AChE: repeated-measures-ANOVA, *df* = 4, x^2 = 8.59, p > 0.05).

Table 3: Enzymatic activity rates (µmol/mg protein/min^{-1}) for the studied biomarkers (GST, GR, AChE), for all treatment groups after exposure to the single fungicide formulation Vivando®.

Biomarker	GST			GR		
	Control	Dermal	Oral	Control	Dermal	Oral
Day 0	219.65 ± 48.38	193.7 ± 56.66	195.55 ± 45.38	21.49 ± 5.82	24.86 ± 14.2	16.34 ± 7.6
Day 1	201.32 ± 46.76	204.95 ± 46.95	194.63 ± 66.07	18.49 ± 4.88	19.32 ± 5.07	15.42 ± 5.01
Day 2	208.92 ± 53.79	195.86 ± 55.18	207.94 ± 36.45	18.51 ± 5.21	18.01 ± 9.62	14.95 ± 4.86
Day 3	209.81 ± 36.11	186.8 ± 35.33	203.98 ± 15.9	18.82 ± 4.28	18.4 ± 9.61	15.92 ± 4.84
Day 4	240.23 ± 115.69	201.56 ± 108.32	216.33 ± 107.67	17.82 ± 2.23	20.08 ± 6.13	16.1 ± 5.76

Biomarker	AChE		
	Control	Dermal	Oral
Day 0	0.66 ± 0.22	0.69 ± 0.22	1.26 ± 0.76
Day 1	0.55 ± 0.24	0.61 ± 0.23	0.79 ± 0.28
Day 2	0.48 ± 0.25	0.49 ± 0.19	0.83 ± 0.41
Day 3	0.48 ± 0.24	0.69 ± 0.33	1.28 ± 0.75
Day 4	0.81 ± 0.38	1.08 ± 0.47	1.16 ± 0.31

Enzymatic biomarker activity – Roundup® UltraMax

Enzymatic activities for the studied biomarkers after exposure to Roundup® UltraMax are given in Table 4. No significant changes in biomarker activity were observed for individuals of the control group (GST: repeated-measures-ANOVA, *df* = 4, x^2 = 10.23, p > 0.05 / GR: repeated-measures-ANOVA, *df* = 4, x^2 = 13.31, p > 0.05 / AChE: repeated-measures-ANOVA, *df* = 4, x^2 = 8.49, p > 0.05). Individuals of the dermal exposure group displayed a significant decrease in GST activity during days 1 and 2 after exposure, while a significant

decrease in GR activity was observed during day 3 after exposure (GST: repeated-measures-ANOVA, $df = 4$, $x^2 = 7.27$, $p < 0.05$; Bonferroni post-hoc test days 1 and 2, $p < 0.05$ / GR: repeated-measures-ANOVA $df = 4$, $x^2 = 21.173$, $p < 0.05$; Bonferroni post-hoc test day 3, $p < 0.05$). Also a significant decrease in GST activity was noted for individuals of the oral group during days 2 and 4 after exposure, while a significant decrease in GR activity was identified during days 2 and 3 after exposure to Roundup® UltraMax (GST: repeated-measures-ANOVA, $df = 4$, $x^2 = 16.352$, $p < 0.05$; Bonferroni post-hoc test day 4, $p < 0.05$ / GR: repeated-measures-ANOVA, $df = 4$, $x^2 = 17.025$, $p < 0.05$; Bonferroni post-hoc test days 2 and 3, $p < 0.05$). No effects on AChE activity were detected (dermal group: repeated-measures-ANOVA, $df = 4$, $x^2 = 8.79$, $p > 0.05$ / oral group: repeated-measures-ANOVA, $df = 4$, $x^2 = 10.23$, $p > 0.05$).

Table 4: Enzymatic activity rates (µmol/mg protein/min^{-1}) for the studied biomarkers (GST, GR, AChE), for all treatment groups after exposure to the single herbicide formulation Roundup® UltraMax.

Biomarker	GST			GR		
	Control	Dermal	Oral	Control	Dermal	Oral
Day 0	202.96 ± 78.7	285 ± 79.80	252.12 ± 65.6	24.36 ± 10.06	28.37 ± 12.16	21.58 ± 11.2
Day 1	181.24 ± 118.5	153.59 ± 58.64	238.08 ± 88.65	26.96 ± 17.18	26.32 ± 18.92	20.44 ± 11.3
Day 2	166.94 ± 79.6	215.81 ± 63.42	212.91 ± 32.95	19.86 ± 11.11	16.47 ± 18.78	8.94 ± 3.4
Day 3	239.08 ± 135.4	217.11 ± 90.9	227.24 ± 56.46	21.47 ± 15.96	12.38 ± 17.53	12.01 ± 6.9
Day 4	200.86 ± 74.5	211.33 ± 102.96	179.88 ± 45.24	26.1 ± 18.81	29.69 ± 15.89	30.87 ± 21.3

Biomarker	AChE		
	Control	Dermal	Oral
Day 0	0.73 ± 0.39	0.8 ± 0.57	0.82 ± 0.38
Day 1	0.61 ± 0.41	0.49 ± 0.19	0.94 ± 0.37
Day 2	0.84 ± 0.63	0.59 ± 0.18	0.95 ± 0.49
Day 3	0.65 ± 0.22	0.5 ± 0.3	0.64 ± 0.32
Day 4	0.47 ± 0.23	0.66 ± 0.52	0.47 ± 0.27

Food consumption and Body weight

Food consumption did not differ between exposure groups during days following exposure to any of the tested formulations (Table 5). Regarding changes in body mass, no significant

differences were notable between exposure groups after finishing each exposure trial (Fungicide mix: ANOVA $F_{2, 0.118} = 0.451$, $p > 0.05$ / Vivando®: ANOVA $F_{2, 0.255} = 0.322$, $p > 0.05$ / Roundup® UltraMax: ANOVA $F_{2, 0.323} = 1.499$, $p > 0.05$).

Table 5: Pairwise comparisions (ANOVA) regarding food consumption between exposure groups for all tested pesticide formulations during the days following each exposure trial.

	Day after exposure			
Formulation	1	2	3	4
Mix (Enervin® + Vivando®)	ANOVA $F(2.\ 1.501) = 1.282$, $p > 0.05$	ANOVA $F(2.\ 0.255) = 0.322$, $p > 0.05$	ANOVA $F(2.\ 0.346) = 0.500$, $p > 0.05$	ANOVA $F(2.\ 2.122) = 2.946$, $p > 0.05$
Vivando®	ANOVA $F(2.\ 1.402) = 2.388$, $p > 0.05$	ANOVA $F(2.\ 0.955) = 1.314$, $p > 0.05$	ANOVA $F(2.\ 0.548) = 0.780$, $p > 0.05$	ANOVA $F(2.\ 1.332) = 2.566$, $p > 0.05$
Roundup® UltraMax	ANOVA $F(2.\ 0.402) = 0.436$, $p > 0.05$	ANOVA $F(2.\ 0.653) = 1.353$, $p > 0.05$	ANOVA $F(2.\ 1.292) = 2.302$, $p > 0.05$	ANOVA $F(2.\ 1.126) = 1.946$, $p > 0.05$

Thermoregulation

At 48h, 72h and 96h after exposure to the fungicide mixture (Enervin® + Vivando®), significantly ($p < 0.05$) more individuals of the dermal and oral exposure groups were observed basking, when compared to the control group. No effects on basking behavior were recognized for any treatment group after exposure to Vivando® nor Roundup® UltraMax (Table 6).

Table 6: Comparison of basking behavior (Chi^2-Test) between exposure groups during days following an exposure trial. * = significant difference to day 0.

		Day after exposure			
Formulation	Exposure pathway	1	2	3	4
Mix (Enervin® + Vivando®)	Oral - Control	$X^2 = 0.635$, $df = 1$, $p > 0.05$	$X^2 = 5.104$, $df = 1$, $p < 0.05$*	$X^2 = 6.286$, $df = 1$, $p < 0.05$*	$X^2 = 2.620$, $df = 1$, $p < 0.05$*
	Dermal - Control	$X^2 = 0.9$, $df = 1$, $p > 0.05$	$X^2 = 8.018$, $df = 1$, $p < 0.05$*	$X^2 = 4.917$, $df = 1$, $p < 0.05$*	$X^2 = 0.322$, $df = 1$, $p < 0.05$*
Vivando®	Dermal - Oral - Control	$X^2 = 0.825$, $df = 2$, $p > 0.05$	$X^2 = 1.104$, $df = 2$, $p > 0.05$	$X^2 = 2.298$, $df = 2$, $p > 0.05$	$X^2 = 0.980$, $df = 2$, $p > 0.05$
Roundup® UltraMax	Dermal - Oral - Control	$X^2 = 8.2$, $df = 2$, $p > 0.05$	$X^2 = 2.035$, $df = 2$, $p > 0.05$	$X^2 = 4.8$, $df = 2$, $p > 0.05$	$X2 = 1.367$, $df = 2$, $p > 0.05$

As for the actual temperature measured in basking individuals, lizards of the control group showed visibly lower temperatures than those of exposed groups, when compared to the

ambient temperature within their terraria (Table 7). This trend becomes reflected when observing the p-values for the fungicide mixture (ANOVA $F_{2,\ 8.617} = 2.862$, $p = 0.06$). However, no such trend was observed for the Vivando® and Roundup® applications (Vivando®: ANOVA $F_{2,\ 1.318} = 0.825$, $p > 0.05$; Roundup®: ANOVA $F_{2,\ 1.629} = 0.329$, $p > 0.05$).

Table 7: Average temperature difference of basking individuals when compared to ambient temperature (within terraria) after exposure to pesticides.

	Temperature (°C)		
Group	Mix (Enervin® + Vivando®)	Vivando®	Roundup® UltraMax
Control	0.52	0.63	0.52
Dermal	1.28	0.81	1.79
Oral	1.35	0.97	1.56

Locomotor performance

Running speed

Significant differences in running speed were detected within groups, between days after exposure in individuals of the oral (significantly slower during day 2 and 4) and dermal (significantly slower during days 2, 3 and 4) treatment groups after exposure to the fungicide mixture (Figure 1; dermal group: repeated-measures-ANOVA, $df = 4$, $x^2 = 15.45$, $p < 0.05$; Bonferroni post-hoc test, $p < 0.05$ / oral group: repeated-measures-ANOVA, $df = 4$, $x^2 = 13.789$, $p < 0.05$; Bonferroni post-hoc test, $p < 0.05$). No significant effects on running performance were detected regarding individuals of the control group (repeated-measures-ANOVA, $df = 4$, $x^2 = 21.12$, $p > 0.05$). Regarding between-group effects, an ANCOVA revealed significant differences in running speed between dermal and control groups on days 2, 3 and 4, and between oral and control groups during day 2 after exposure (Tables 8 and 9). Additionally, the analysis revealed that neither individual temperature nor gender, SVL or

body mass had an effect on running speed of individuals, but that it was only affected by treatment group affiliation.

Table 8: Between group differences (ANCOVA) in running performance for exposure groups using gender, body mass, SVL and individual temperature as co-variables. * = significant difference to day 0.

	Day after exposure				
Formulation	0	1	2	3	4
Mix (Enervin® + Vivando®)	ANCOVA $F(6, 0.484) = 1.485$, $p > 0.05$	ANCOVA $F(6, 0.552) = 1.247$, $p > 0.05$	ANCOVA $F(6, 1.759) = 3.294$, $p < 0.05$ *	ANCOVA $F(6, 1.754) = 2.999$, $p < 0.05$ *	ANCOVA $F(6, 2.586) = 4.854$, $p < 0.05$ *
Vivando®	ANCOVA $F(6, 0.418) = 0.904$, $p > 0.05$	ANCOVA $F(6, 0.418) = 0.904$, $p > 0.05$	ANCOVA $F(6, 0.482) = 0.581$, $p > 0.05$	ANCOVA $F(6, 0.820) = 0.781$, $p > 0.05$	ANCOVA $F(6, 0.902) = 1.354$, $p > 0.05$
Roundup® UltraMax	ANCOVA $F(6, 0.477) = 1.543$, $p > 0.05$	ANCOVA $F(6, 0.478) = 1.281$, $p > 0.05$	ANCOVA $F(8, 0.901) = 1.776$, $p > 0.05$	ANCOVA $F(8, 0.738) = 1.964$, $p > 0.05$	ANCOVA $F(8, 0.283) = 0.527$, $p > 0.05$

Table 9: Post-hoc test (Bonferroni) to detect significant differences in climbing performance between exposure treatments for significant ANCOVA results. * = significant difference to day 0.

	Control				
Post-hoc Test (Bonferroni)	Day after exposure				
Group	0	1	2	3	4
Dermal	$p > 0.05$	$p > 0.05$	$p < 0.05$*	$p < 0.05$*	$p < 0.05$*
Oral	$p > 0.05$	$p > 0.05$	$p < 0.05$*	$p > 0.05$	$p > 0.05$

For the single fungicide formulation Vivando®, no effects could be attested, neither within (Figure 2, Table 8; control: repeated-measures-ANOVA, $df = 4$, $x^2 = 15.19$, $p > 0.05$ / dermal: repeated-measures-ANOVA, $df = 4$, $x^2 = 12.45$, $p > 0.05$ / oral: repeated-measures-ANOVA $df = 4$, $x^2 = 8.56$ $p > 0.05$), nor between treatment groups (Figure 2, Table 8).

As for Roundup® Ultramax, neither differences in running speed were observed within exposure groups during the days after exposure (control: repeated-measures-ANOVA, $df = 4$, $x^2 = 15.55$, $p > 0.05$ / dermal: repeated-measures-ANOVA, $df = 4$, $x^2 = 11.62$, $p > 0.05$ / oral: repeated-measures-ANOVA, $df = 4$, $x^2 = 2.34$, $p > 0.05$) nor between groups, either (Figure 3, Table 8).

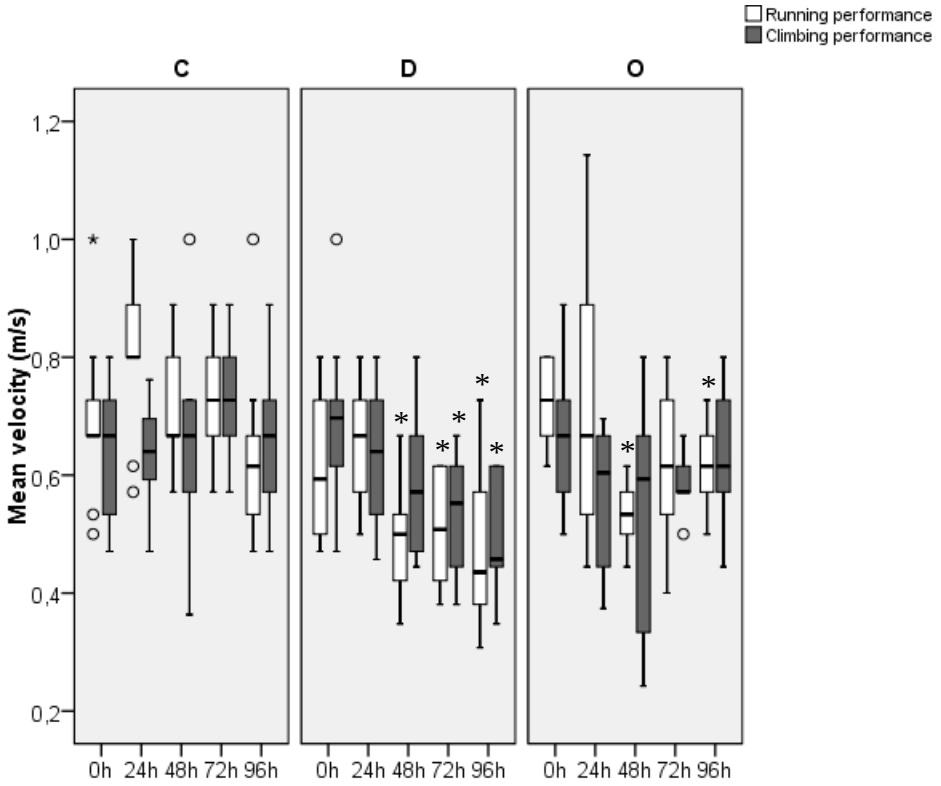

Figure 1: Mean running (white) and climbing (grey) performance (m/s) after exposure to the fungicide mixture (Enervin® + Vivando®) between treatment groups. C = control group, D = dermal group, O = oral group. * = significant difference to 0h within each respective treatment group.

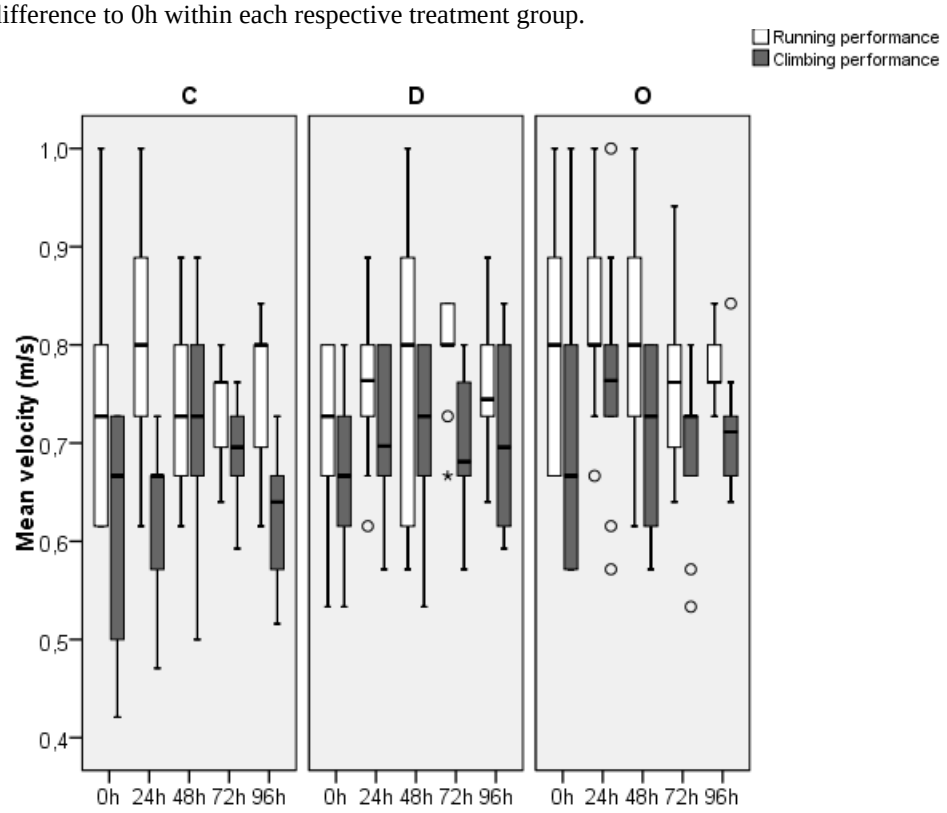

Figure 2*:* Mean running (white) and climbing (grey) performance (m/s) after exposure to Vivando® between treatment groups. C = control group, D = dermal group, O = oral group.

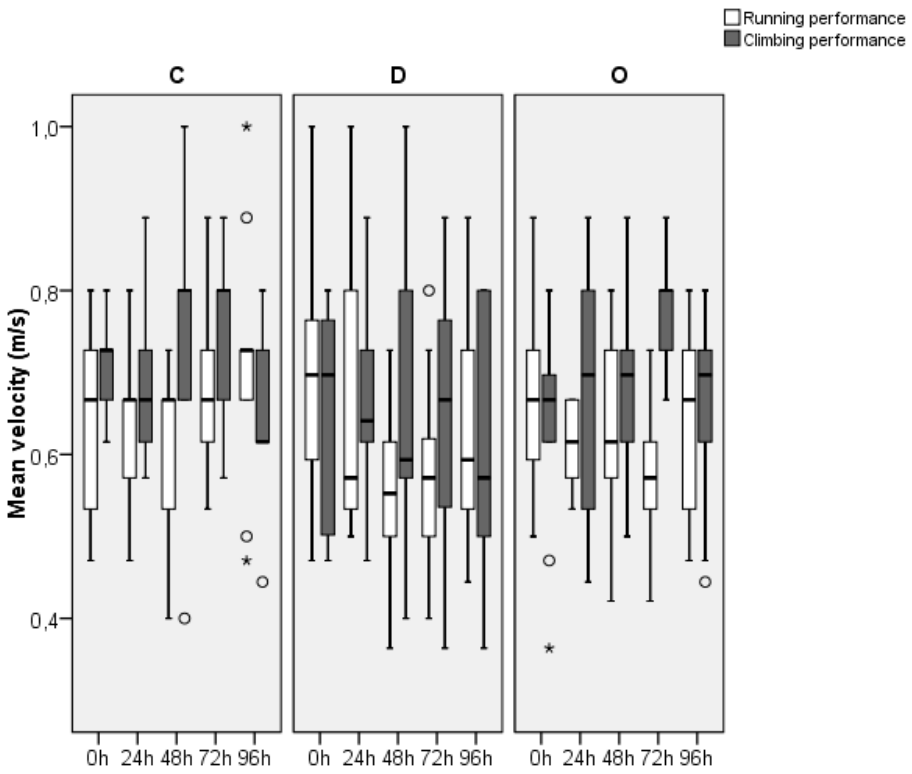

Figure 3: Mean running (white) and climbing (grey) performance (m/s) after exposure to the Roundup® UltraMax between treatment groups. C = control group, D = dermal group, O = oral group.

Climbing speed

After exposure to the fungicide mixture, a significant decrease in climbing speed was observed in individuals of the dermal treatment group during days 3 and 4 after exposure (repeated-measures-ANOVA, $df = 4$, $x^2 = 5.65$, $p < 0.05$, Bonferroni test, $p < 0.05$), but not within oral and control groups (repeated-measures-ANOVA, $df = 4$, $x^2 = 17.47$, $p > 0.05$ and repeated-measures-ANOVA, $df = 4$, $x^2 = 21.59$, $p > 0.05$, respectively; Figure 1). Individuals of the control group were significantly faster than those of the dermal and oral treatment groups on day 3 after exposure, and faster than individuals of the dermal group on day 4 (Tables 10 and 11). Again, out of all relevant co-variables, only group affiliation had a significant effect on climbing performance.

For individuals exposed to Vivando® only, no effects were detected within treatment groups (control: repeated-measures-ANOVA, $df = 4$, $x^2 = 14.93$, $p > 0.05$ / dermal: repeated-measures-ANOVA, $df = 4$, $x^2 = 6.92$, $p > 0.05$ / oral: repeated-measures-ANOVA, $df = 4$, $x^2 = 21.47$, $p > 0.05$). No differences in climbing performance were found between groups, either (Figure 2, Table 10).

Table 10: Between group differences (ANCOVA) in climbing performance for exposure groups, using gender, body mass, SVL and individual temperature as co-variables. * = significant difference to day 0.

	Day after exposure				
Formulation	0	1	2	3	4
Mix (Enervin® + Vivando®)	ANCOVA $F(6, 0.134) = 0.318$, $p > 0.05$	ANCOVA $F(6, 0.334) = 0,578$. $p > 0.05$	ANCOVA $F(6, 5.427) = 1.692$, $p > 0.05$	ANCOVA $F(6, 1.527) = 7.640$, $p < 0.05$ *	ANCOVA $F(6, 1.722) = 3.306$, $p < 0.05$ *
Vivando®	ANCOVA $F(6, 2.419) = 2.320$, $p > 0.05$	ANCOVA $F(6, 1.542) = 2,148$. $p > 0.05$	ANCOVA $F(6, 0.370) = 0.416$, $p > 0.05$	ANCOVA $F(6, 21.427) = 1.320$, $p > 0.05$	ANCOVA $F(6, 0.319) = 0.234$, $p > 0.05$
Roundup® UltraMax	ANCOVA $F(6, 0.959) = 2.709$, $p > 0.05$	ANCOVA $F(6, 0.470) = 1,677$. $p > 0.05$	ANCOVA $F(6, 0.724) = 0.416$, $p > 0.05$	ANCOVA $F(6, 0.659) = 1.422$, $p > 0.05$	ANCOVA $F(6, 0.735) = 1.202$, $p > 0.05$

Similarly, no effects on climbing performance were recorded within groups after exposure to Roundup® UltraMax (control: repeated-measures-ANOVA, $df = 4$, $x^2 = 23.21$, $p > 0.05$ / dermal: repeated-measures-ANOVA, $df = 4$, $x^2 = 16.22$, $p > 0.05$ / oral: repeated-measures-ANOVA, $df = 4$, $x^2 = 18.69$, $p > 0.05$) or between groups (Figure 3, Table 10).

Table 11: Post-hoc test (Bonferroni) to detect significant differences in climbing performance between exposure treatments for significant ANCOVA results. * = significant difference to day 0.

	Control				
Post-hoc Test (Bonferroni)	Day after exposure				
Group	0	1	2	3	4
Dermal	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p < 0.05$*	$p < 0.05$*
Oral	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p < 0.05$*	$p > 0.05$

Discussion

Enzymatic biomarkers

Concerning the main goal of the study - i.e. the validation of saliva sampling as a reliable method to detect pesticide exposure in reptiles using enzymatic biomarker assays - we were able to confirm the suitability of the sampling method to detect alterations in biomarker activity. As opposed to our previous field studies (Mingo et al. 2017a,b), potential interference of other environmental parameters causing unaccounted synergisms or antagonisms can be discarded. Testing individuals from different populations like in Mingo et al. might also bring alternating enzymatic activity caused by differences in population genetics. In the present study, these effects can be excluded, as all individuals were sampled from within one population. The fact that in the present laboratory study exposure groups were compared to an actual control group, instead of individuals of the same population prior to any application, provides further evidence backing up the suitability of the approach. In this study, no changes occurred within control groups during any exposure trial, in contrast to exposed treatment groups.

Overall, results were similar to those obtained in our field studies. Individuals exposed to the fungicide mixture displayed a biomarker activity peak at around day 2 after exposure for GST and GR. As previously reported, there don’t seem to be any neurotoxic effects caused by the applied fungicide formulations (Mingo et al. 2017a,b).

No effects were observed in individuals of any treatment group after being exposed to the single fungicide formulation Vivando®. This fungicide was chosen because of the comparatively low quantities that are applied in field (0.4l/ha compared to 4kg/ha Enervin® or other formulations, for example; Mingo et al, 2017a,b, BVL 2017). The goal of this

specific trial was to find out whether such low quantities actually have an observable impact on individuals detectable by using of buccal swabs. Apparently, this is not the case. Given that within vineyards, fungicides were always applied in a mix of two or more formulations at a given time (Mingo et al. 2017a,b, BVL 2017), more importance should probably be given to the effects measured for the fungicide mix application. Unfortunately, no studies have been conducted so far using pesticide mixtures as test substance and a squamate reptile species as test organism. Comparing with results gained in amphibian toxicology, Mesléard et al. (2016) tested the impact of an insecticide (alphacypermetrine) and an herbicide (oxadiazion) on survival of prometamorphic larvae of the green frog *Pelophylax perezi*. Because the pesticides are usually applied in combination – similar to the fungicide mix used in our vineyards – the authors tested both substances singly and in combination. They found a highly deleterious impact of their combined use (nearly 100% mortality) on survival until metamorphosis, but also a measurable impact of both substances used separately (about 30-40% mortality, nearly 100% survival in the control). Taking these results as an example, one may suggest that in the present study, Enervin® contributed most to the effects of the pesticide mixture because Vivando® induced no effect at all in lizards. However, synergistic effects of pesticides used in combination are described in amphibian-toxicological studies (e.g. of a mix of a glyphosate-based and a dicamba-based herbicide on the induction of primary DNA breaks on circulating blood cells of *Rhinella arenarum* larvae: Soloneski et al. 2016). Such effects cannot be ruled out for effects on *P. muralis* of the fungicide mixture in the present study, compared to Enervin® in single use. Similarly, Güngördü et al. (2016) reported synergistic effects of glyphosate- and methidathion-based pesticides in tadpoles of three amphibian species when administered as a mix. This resulted in significantly increased detoxification responses when compared to the single formulations.

The exposure to the glyphosate formulation Roundup® UltraMax led to a clear inhibition in GR and GST activity. This stands in contrast to previous field data, where exposure to glyphosate-based herbicides generally induced a strong GST and GR response. In the case of the Touchdown® formulation used in the field, there was even evidence hinting towards potential neurotoxicity (Mingo et al. 2017a). However, this may easily be explained by the fact that we used a different formulation (Roundup® UltraMax) than those applied during field trials (Touchdown®: Mingo et al. 2017a, Clinic Ace®: Mingo et al. 2017b). It is doubtful whether these effects are actually caused by glyphosate itself, especially considering the differing effects between formulations, but by the adjuvants used in said formulations, which in many cases remain unknown (Cox and Surgan 2006, Boone et al. 2014) and differ from formulation to formulation (Wagner et al. 2013). Concerning the inhibition of GST, Lajmanovich et al. (2011) detected very similar results whilst studying the effects of different herbicide formulations on enzymatic activities in the toad *Rhinella arenarum*, one of which was Roundup® UltraMax. This same response was further observed in fish of the genus *Rhamadia* after exposure to the same formulation (de Menezes et al. 2011). According to de Menezes et al. (2011), this suggests a failure in detoxification during the exposure period, which they linked to an increase of oxidative stress. During the recovery period however, the authors observed an increase in GST activity, which could be seen as a compensatory response to detoxify tissues. Indeed, such an effect was observed for GR at 96h after exposure to Roundup®. For GST however, this time period seemed to be too short to observe such a response.

Generally speaking, effects observed between both exposed treatment groups (dermal and oral) were very similar. Dermal and oral exposure are considered the main uptake routes of pesticides in reptiles (Hopkins 2006, Salice and Weir 2011, Sparling et al. 2010, Todd et al.

2010, Weir et al. 2014, 2015). Therefore, exploring whether saliva samples are suitable to detect both exposure pathways separately seemed rather interesting. While phase II metabolism, to which GR and GST belong to, generally comprises a systemic response once distribution to blood and tissues has occurred (Shargel and Yu 2016), it stands to reason that a response within the buccal cavity could be higher for orally dosed individuals, since there is direct contact between the analyzed tissue fraction and the xenobiotic substance, as opposed to dermal uptake.

Yet, there seems to be a trend towards stronger oxidative stress caused by dermal uptake. This effect may be explained by a more or less "selective" exposure of individuals of the oral treatment, as uptake solely depended on food intake. While initially strong, exposure greatly diminishes as individuals have satisfied their energetic needs (resulting in a high uptake at the beginning of the experiment, but comparatively low uptake onwards). Given that wall lizards are poikilothermic, this assumption seems plausible (Avery 1978, Nagy et al. 1999, Schulte 2008). However, being confined to terraria, it can be expected that the actual energy expenditure of individuals was lower than *in situ*, meaning that food intake will be probably higher under natural conditions (Avery 1978). In case of dermal treatment groups, exposure of individuals was 'maximal', with no way of avoidance, as lizards were directly over-sprayed, and probably kept being exposed through soil contact (Van Meter et al. 2015). Weir et al. (2014) reported that, when exposed to the same pesticide concentrations, oral and dermal exposure resulted in similar residue levels within the Western fence lizard (*Sceloporus occidentalis*). Thus, while detoxification through the oral pathway is probably more punctual, the same may not apply for dermal exposure, potentially resulting in higher body burdens, and thus, in increased oxidative stress when compared to oral exposure. Whether different

exposure routes are over- or underrepresented using this method therefore needs to be further studied.

In our previous work, significance extended to more days after exposure. However, this is probably caused by the difference in sample size between studies, which because of conservation and animal welfare concerns, only amounted to 10 individuals per treatment group (Freedman et al. 2007). Furthermore, we only tested single exposure pathways. Under field conditions, however, individuals can, in many cases, be expected to be exposed via both uptake pathways simultaneously, most probably increasing effects as compared to only one pathway (Hopkins 2006, Sparling et al. 2010, Todd et al. 2010, Salice and Weir 2011).

No effects on food consumption and body mass

The lack of differences in food consumption rate (and consequently the lack of differences in body mass) between treatment groups, for any of the tested formulations, indicates that there is (1) no avoidance of contaminated prey and (2) no substance-induced feeding apathy (EFSA 2004). Otherwise, lizards of the oral treatment group should have displayed a lower food consumption rate (Pascual et al. 1999, EFSA 2004). However, studying avoidance under laboratory conditions is a rather complicated matter, as a lack of avoidance does not necessarily imply that under natural conditions, individuals may not shift towards non-contaminated prey when available (EFSA 2004, 2009). The observed lack of avoidance may therefore simply be conditioned by the lack of alternatives. Under natural conditions, lizards cannot be expected to only prey from directly treated area (vineyard) itself, but will use different microhabitats which will in turn alter uptake intensity (Duelli 1990, Walklate 1992, Schulte 2008, EFSA 2004). For instance, Yanes-Marichal et al. (2017) observed differences in acceptance/avoidance of pesticide treated and untreated food items between individuals of

the Tenerife lizard (*Gallotia galloti*), depending on whether they were retrieved from a natural site or a cultivated site with regular pesticide applications. Here, lizards from the untreated sites seemed to discriminate between contaminated and uncontaminated food items, avoiding the former. However, lizards captured at the cultivated sites did not differentiate between food items. This may imply that lizards living within treated areas may be accustomed to contaminated food, and do not differentiate anymore. While the wall lizards tested within this study originated from an untreated site, orally exposed individuals were not able to choose between food items. Whether avoidance may take place under field conditions, and if it does, to which degree, remains to be further explored.

Effects on thermoregulation

Exposure to the fungicide mixture resulted in a clearly higher basking activity of individuals of the oral and dermal treatment. So called 'fever responses' have been observed in reptiles before (Carpenter et al. 2016) and can be explained by an increase of metabolic rate of individuals as a means of detoxification, due to their poikilothermic character (Talent 2005, Schulte 2008). This increase in basking activity did not result in significant differences in body temperature amongst treatment groups, however. In case of the fungicide mixture, this may be explained by the comparatively low amount of tested individuals, as the p-value was near significance ($p = 0.06$).

The lack of differences in basking behavior concerning the formulation Vivando® may once again be explained by the low concentration of the application. In the case of Roundup® UltraMax, no significant differences regarding the amount of basking individuals could be observed between groups, although, similar to the fungicide mixture exposure, the mean temperature of basking individuals was about 1°C higher than in the control group. Yet,

statistical significance was not achieved. According to these results, exposure to fungicide mixtures, as commonly applied in viniculture (BVL 2017) could have strong implications for reptile wildlife, as a prolonged basking activity will probably increase predatory pressure on individuals, potentially leading to increased mortality. In fact, our previous study (Mingo et al. 2017b) revealed that populations of the Common Wall lizard in agricultural landscapes are characterized by differing population structures along an agricultural gradient. Specifically, age structure drastically differed to that of the reference population, with many less 'older' individuals with increasing exposure intensity, indicating increased mortality.

Effects on locomotor performance

Running and climbing performance was only significantly affected after exposure to the fungicide mixture, but not after exposure to Vivando® nor Roundup® UltraMax. There may be multiple reasons for these observed changes in locomotor performance. Generally speaking, these kind of tests have been employed to detect potential neurotoxic substances, affecting AChE or other neurotransmitters (DuRant et al. 2007). However, none of the tested formulations did have any effect on AChE activity. Possible interference with other neurotransmitters is possible (Vaccari et al. 1999, Lionetto et al. 2013, Strelitz et al. 2014), although we cannot confirm it, as we lack the data to address this topic. However, the cause of these impairments may not be related to neurotoxicity at all. Concerning the data gained from biomarker activities and behavioral observations, it stands to reason that this may very well be caused by increased metabolic rates and oxygen consumption, as a side effect of detoxification. Toxicant metabolism has an additional energetic cost, which can influence the metabolic and energetic investment of individuals, thus compromising other biological functions (Talent 2005, Halsey and White 2010). Amaral et al. (2012b) detected that Bocage's wall lizards (*Podarcis bocagei*) inhabiting sites with regular pesticide treatments appeared to

have elevated oxygen consumption when compared to animals of reference locations (up to 32% higher). This may lead to a decrease in energy available for other biological functions, and may be the cause for a reduction in mobility. However, our individuals were retrieved from a non-exposed population. Whether the increased oxygen consumption and metabolic rate observed in *Podarcis bocagei* is a way of adaptation or may be caused by short term exposure to pesticides is unknown. Additionally, Amaral et al. (2012b) did not observe any significant differences in locomotor performance between populations.

Another possibility is that the observed impairments in locomotor performance are directly caused by the active ingredients, the surfactants (Cox and Surgan 2006, Wagner et al. 2013, Boone et al. 2014) or synergistic effects of both fungicides (Soloneski et al. 2016). Fungicides are often considered to have general biocidal properties regarding different taxonomic groups. This is because of their mode of action, which is often not fungi specific. For example, many fungicides target energy production or cell division, processes which are highly conserved throughout all taxa. In consequence, targeting these processes will be toxic to a wide range of organisms (Maltby et al. 2009).

This also applies to Enervin®, the major component of the fungicide mixture, which consists of the active ingredients Initium® and Metiram® (i.e. even Enervin® singly used is strictly spoken a mix), both of which affect energy production of the target organism.

Initium® targets complex 3, a membrane protein complex present in the mitochondria of all animals (Mitchell 1975, Gao et al. 2003), and impairs the electron transport in the respiratory chain of the pathogen, thus making it unable to generate the energy required for keeping the organism alive. Metiram® affects the Pyruvate dehydrogenase complex, which is essential for the energy production from carbohydrates (Izard et al. 1999).

Based on the data at hand, it is difficult to discern which the most probable cause for observed impairments in locomotor performance may be (i.e. increased energetic expenditure, non-observed neurotoxicity, or the active ingredients).

The lack of significance during the Roundup® UltraMax trials may be an indicator towards an effect indeed caused by the active ingredients of the fungicides, since the active substance of Roundup® (glyphosate) targets chloroplasts of plants (Steinrücken and Amrhein 1980). A direct effect of this component can therefore be discarded. At the same time, adjuvants of this formulation didn't seem to have an effect on lizards either (Cox and Surgan 2006, Wagner et al. 2013, Boone et al. 2014). However, in order to be able to thoroughly answer this question, further research is needed.

Finally, it seems that individuals of the dermal treatment group were affected more strongly than those of the oral treatment. As mentioned before, this may be explained by "avoidable" exposure through food intake, which is punctual, in lower doses, and mostly limited to the beginning of the trial. Incidentally, the decrease in locomotor performance was almost identical to biomarker peak activity in individuals orally exposed to the fungicide mixture, while it continued deteriorating with time within the dermal group. It is not out of the ordinary to assume that biomarker responses will be higher in locally exposed tissue, even though a systemic reaction is triggered (Shargel et al. 2016). Thus, while dermal exposure can be assessed via buccal swabbing, it may underrepresent the effect on the organism when compared to the oral pathway.

Conclusions

We were able to validate the use of buccal swabs as a reliable method to detect pesticide exposure using different enzymatic biomarkers in reptiles. Given that any additional stressors

which may have interfered in previous field studies were eliminated in this laboratory approach, it can be concluded that results gained in previous studies were indeed caused by pesticide exposure and absorption of these xenobiotics in wild lizards. The similarity between obtained field results and the current study is remarkable. However, we still do not know how saliva samples/buccal swabs fare in comparison to traditional blood and organ sample analysis. Both matrices can be expected to be more sensitive, but at the same time much more invasive. Buccal swabs do represent a good method to explore exposure and potential effects of pesticide formulations in reptiles. Due to its minimal-invasiveness, it allows to test a much higher amount of individuals, opening new possibilities for study designs.

Tests of locomotor performance seemed to imply that exposure to fungicide mixtures (as commonly applied in viniculture) can significantly impair mobility of individuals, which may entail potentially severe repercussions for reptile wildlife, as it could reduce survival of lizards targeted by predators. However, the exact mechanisms of action leading to this effect remain unclear. Given the data at hand, a decrease in energy reserves caused by increased metabolic rates or the direct effect of active ingredients seem a reasonable explanation. However, further research is needed in order to detect whether impairments in locomotor performance also occur under field conditions. At the same time, individuals exposed to a fungicide mixture containing the formulations Enervin® and Vivando® displayed longer basking activities, also making them more susceptible towards potential predation.

Being non-target organisms of pesticide applications, consequences of exposure in reptiles need to be further studied and comprehended, especially as their relevance for pesticide admission procedures in the EU will increase in the near future. Thus, new ways to detect effects and impacts on populations, as well as development of new methods and study designs are urgently needed.

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CHAPTER III

Determination of pesticides adsorbed on arthropods and gastropods by a micro-QuEChERS approach and GC-MS/MS

Markus Stöckelhuber[1], Christoph Müller[1], Florian Vetter[1], Valentin Mingo[2], Stefan Lötters[2], Norman Wagner[2] and Franz Bracher[1]

[1] Department of Pharmacy - Center for Drug Research, Ludwig-Maximilians University Munich, Butenandtstrasse 5-13, 81377 Munich, Germany

[2] Department of Biogeography, Trier University, Universitätsring 15, 54296 Trier, Germany

Abstract

A miniaturised QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Save) approach combined with gas chromatography-tandem mass spectrometry (GC-MS/MS) has been developed for the simultaneous determination of nine pesticides (Cyflufenamide, Difenoconazole, Dimethomorph, Fluopicolide, Fluopyram, Metrafenone, Myclobutanil, Quinoxyfen, and Tebuconazole) in insects, snails, and spiders. In contrast to the original QuEChERS approach, only 500 mg of dried and homogenised sample matrix, mixed with 1.0 mL ethyl acetate and 250 mg $MgSO_4$:NaCl (4:1), is required for this novel "micro QuEChERS" protocol. The organic phase was cleaned using dispersive solid-phase extraction (dSPE) with 75 mg $MgSO_4$:PSA sorbent (4:1). The method was validated according to SANCO/12571/2013 and applied to real samples (n = 7). Fluopicolide was the only detectable pesticide in real samples from vineyards. In two samples, the Fluopicolide levels were between the determined LOD and LOQ (0.15 – 1.00 mg kg^{-1}), and in one sample a concentration of 1.68 mg kg^{-1} was detected.

Introduction

Pesticides are widely applied in the production of foods to control the growth of weeds and fungi or to prevent crop damage by insects, mites, rodents, and other pests [1]. In the last 13 years, a multiresidue analytical method for the determination of pesticides, the QuEChERS (Quick, Easy, Effective, Cheap, Rugged, and Save) approach, has become very popular for analysis of fruits and vegetables [2–4]. The QuEChERS methodology has been applied on diverse food matrices [5, 6]. In contrast, only very few methods have been described in the literature dealing with pesticide analysis in arthropods and gastropods. Stahlschmidt and Brühl [7] analysed Chloropyrifosmethyl and Fenoxycarb in arthropods by a QuEChERS LC–

MS/MS approach using 1 g sample size and 10 mL acetonitrile for extraction, and Niell et al. [8] analysed 20 pesticides in bees using a QuEChERS LC–MS/MS approach using 2 g sample size and 10 mL acetonitrile. However, miniaturization of the sample preparation is absolutely necessary in case of very small available sample sizes. Additionally, miniaturization is popular in the field of analytical chemistry [9], because of its advantages like reduced solvent costs, easier handling, storage, and processing.

Arthropods and gastropods are main prey items for various vertebrate groups, and pesticide use can affect the predators mainly in two ways: (1) available food is reduced and (2) contaminated prey items represent a pesticide exposure way. In reptiles, similar to other vertebrate groups, ongoing worldwide population declines are recognized. While the causes for these declines are highly assorted, it is believed that habitat loss and degradation, coupled with environmental pollution (especially in form of pesticides) are the leading factors for these declines, in industrialized countries. Although effects of pesticides on reptiles have been reviewed to some degree, and different studies have shown evidence of potential strong effects on reptile wildlife, toxicity data concerning squamate reptiles (lizards and snakes) is still scarce, and data on effects of pesticides in species' natural habitats even more so [10]. Although many reptile species within the European Union are strictly protected in all member states thanks to the Habitats Directive, detrimental effects of pesticide use are possible within [11] and especially outside special areas of conservation [10]. This is very alarming as negative effects, which could potentially lead to a regional diversity loss, have already been identified in laboratory and mesocosm studies. In order to evaluate the exposure risk of the Common wall lizard (*Podarcis muralis*), the residue unit dose of its prey animals (especially insects, snails, and spiders) at different times after exposure to pesticides has to be determined. Cyflufenamide, Difenoconazole, Dimethomorph, Fluopicolide, Fluopyram,

Metrafenone, Myclobutanil, Quinoxyfen and Tebuconazole were used in the present work, as these pesticides were published in the spray plans of our surveyed vineyards, a typical habitat of *P. muralis*.

The aim of this study was to develop a simple, efficient and rapid method for the analysis of pesticides in the prey animals of P. muralis. The method was fully validated according to SANCO/12571/2013 [12]. The developed method was applied to the analysis of pesticides in real samples. The method development is the corner stone for the analysis of nine selected pesticides in the prey of P. muralis in our observed vineyards, and the obtained results will finally help to assess the oral exposure risk of *P. muralis*.

However, compared to the plant material and food items previously investigated for pesticide content, prey animals of P. muralis possess a completely different tissue composition. Further, only very limited amounts of contaminated animals were available. In consequence, the classical QuEChERS method [2] had to be adapted to the current matrix as well as miniaturized.

Solving the problem with minimal available tissue material (500–1000 mg of dead prey animals collected from vineyards after pesticide exposure), a so-called “micro-QuEChERS” approach was developed and combined with the powerful gas chromatography triple-quadrupole system (GC–MS/MS). This combination allows the simultaneous analysis of pesticides in animal tissue with minute amounts of sample. While most of the QuEChERS methods use acetonitrile as extraction solvent [1–3, 5, 7, 8], we obtained better recoveries and more intense signals by using ethylacetate [4–6, 13]. Also different mixtures of dispersive solid-phase extraction (dSPE) material were tested. The best mixture was 75 mg MgSO4:PSA sorbent (4:1).

Experimental

Materials

Analytical standards were purchased from High Purity Compounds (Cunnersdorf, Germany) and had a purity of ≥98.9%. Pesticide stock solutions were prepared by weighing 10 mg of pure standard and dissolving each compound in 1 mL ethyl acetate. These stock solutions were diluted in 1:10 steps to the final test concentrations and stored at −18 °C. Ethyl acetate in HPLC grade was obtained from VWR International (Darmstadt, Germany). The bulk sorbents C18EC and primary secondary amine (PSA), as well as QuEChERS dSPE EMR-Lipid® (unspecified composition), and QuEChERS Final Polish EMR-Lipid® (MgSO4:NaCl (4:1)) were purchased from Agilent Technologies (Santa Clara, CA, USA). Anhydrous MgSO4 was obtained from Grüssing GmbH (Filsum, Germany).

Laboratory apparatus

To maintain a constant sample weight (humidity) the sampleswere stored in a laboratory drying cabinet at 60 °C for 24 h from WTB Binder Labortechnik (Tuttlingen, Germany). Homogenization of the samples during sample preparation was accomplished by using a Vortex-Genie 2 from Scientific Industries (Bohemia, NY, USA). Separating steps were carried out using a centrifuge 5415 D from Eppendorf (Hamburg, Germany).

Analytical instruments

All pesticide samples were analysed with a GC–MS/MS instrument (3800 series) from Varian (Darmstadt, Germany), utilizing the Varian Workstation 6.9 SP1 software. The injector was a Varian split/splitless injector 1177. The injector was used in splitless mode (for 1 min) at 280 °C. Chromatographic separation was performed on a ZB-5ms column from Phenomenex

(Aschaffenburg, Germany), length 30 m, inner diameter 0.25 mm, film thickness 0.25 µm, with a 10 m column guard. Helium 5.0 was used as a carrier gas with a constant flow rate of 1.3 mL min^{-1}. The initial GC oven temperature was 50 °C (for 1 min) followed by a ramp of 40 °C min^{-1} up to 210 °C, then ramped at 15 °C min^{-1} up to 280 °C, and finally ramped at 20 °C min^{-1} to 310 °C (hold time 6 min). The total run time was 17.2 min. Automated sample injection was accomplished using a CombiPal autosampler from CTC Analytics (Zwingen, Switzerland). The injection volume was 2 µL. The Varian 1200 triple-quadrupole (MS/MS) was operated in EI mode (70 eV), and the collision gas was Argon 4.5. The working conditions were: ion source 250 °C, manifold 40 °C, transfer line 250 °C, and multi-reaction monitoring (MRM) mode (Table 1).

Table 1: GC-MS/MS acquisition parameters with ion transitions, collision energy (CE), and results of the validation with LOQ, LOD, and r^2; Q1 = precursor ion, Q3 = product ion.

Pesticide	Quantitative MRM			Confirmation MRM			Retention time [min]	LOQ [mg kg-1]	LOD [mg kg-1]	Linearity (r^2)
	Q1	Q3	CE [V]	Q1	Q3	CE [V]				
Cyflufenamide	223	203	20	118	90	30	9.5	0.50	0.20	0.993
Difenoconazole	323	265	30	325	267	30	14.9	0.50	0.05	0.998
Dimethomorph	301	165	25	387	301	30	16.0	0.50	0.05	0.993
Fluopicolide	209	182	30	173	109	35	10.7	1.00	0.15	0.995
Fluopyram	173	145	30	396	223	10	8.9	0.20	0.10	0.999
Metrafenone	377	362	30	379	364	30	11.9	0.20	0.15	0.998
Myclobutanil	179	125	30	150	123	30	10.0	0.20	0.10	0.999
Quinoxyfen	237	208	40	272	237	20	10.7	0.50	0.02	0.996
Tebuconazole	250	125	20	125	89	10	11.0	0.50	0.15	0.997

Samples

For the simulation of a blank sample matrix in the course of method development, a fish food mix of dried grubs of Tenebrio molitor (mealworm), Bombyx mori (silkworm), Gammarus fossarum (amphipod freshwater crustacean), and orthopterans (grasshoppers, crickets) was purchased from a local pet shop. The blank sample matrix was analysed (n = 6) using the

newly developed method to determine carry-over effects. None of the nine pesticides were present in the blank sample matrix.

Prey animals (insects, snails, and spiders) of P. muralis were collected using a ‘bug catcher’ 0, 1, and 3 days after pesticide exposure in three vineyards around Trier (Germany) and frozen at −80 °C until further analysis. Due to the fact that not enough sample material of each animal species was available on each day and vineyard, all animals were blended to one sample which represents one day from one vineyard.

Sample preparation

The samples were dried at 60 °C for 24 h and then homogenized with a mortar and pestle. Dried samples (500 mg) were weighed into 2 mL microcentrifuge tubes and mixed with 1.0 mL ethyl acetate. The mixture was manually shaken for 1 min, then 250 mg of QuEChERS Final Polish EMR-Lipid® (MgSO4:NaCl (4:1)) were added, followed by shaking for another 1 min. The sample was centrifuged for 5 min at 9500 xg at room temperature. Three hundred microliter of the supernatant was transferred into a 1.5 mL microcentrifuge tube containing a mixture of 75 mg anhydrous MgSO4:PSA (4:1). The tube was shaken for 1 min and centrifuged again for 5 min at 9500 xg at room temperature. The supernatant was transferred into a 0.15 mL insert and analysed by GC–MS/MS.

Results and Discussion

Sample Clean-up Optimization

Evaluation of the extraction solvent

In this study, we tested both commonly used QuEChERS extraction solvents acetonitrile and ethyl acetate [1–6, 8, 13]. At first glance, extracts obtained with acetonitrile were nearly colourless, whereas ethyl acetate extracts showed a yellow tint. This suggests an extraction of different groups of substances and especially a different clean-up efficiency, but it is also important to emphasize that it is not possible to determine accurately that one extract has less co-extracted compounds than another just by observing the colour of the extracts [14, 15]. The obtained recoveries (data not shown) of the pesticides (for each condition n = 6) in ethyl acetate and acetonitrile were comparable for all dSPE mixtures (n = 3, see below), but acetonitrile involved some disadvantages. Extracts prepared using acetonitrile gave significantly smaller signal areas than extracts prepared using ethylacetate, proved with a t-test with a significance level of 95%. Moreover, acetonitrile has a larger solvent expansion during vaporization in the inlet of the GC–MS/MS system, is more expensive and more toxic than ethylacetate [2]. For all of these reasons ethyl acetate was selected over acetonitrile as extraction solvent.

Evaluation of dSPE sorbents

One of the main advantages of the QuEChERS methodology is the use of a dSPE step which gives the possibility to easily adapt the type and amount of dSPE sorbents (e.g. C18, GCB (graphitized carbon black), MgSO4, and PSA) used to the current matrix [8]. This and the low amount of time required for sample workup is the reason why the QuEChERS method has

been successfully applied to many different types of matrices [14]. The extraction step with ethyl acetate and 250 mg MgSO4:NaCl (4:1) already led to recoveries near 100%, so the sorbents used in the extraction step have not been further investigated. For the clean-up step different mixtures of 75 mg PSA:MgSO4, (1:4), PSA:C18EC:MgSO4 (1:1:4) and Agilent's QuEChERS dSPE EMR-Lipid® have been tested. For this purpose, the blank sample matrix (fish food mix) was spiked with each 1.50 mg kg−1 of the pesticides. MgSO4 was used in every dSPE sorbent mixture to remove residual water from the organic phase to facilitate GC–MS/MS analysis. PSA is known to remove many impurities like fatty acids, polar pigments, and sugars from the extract [2, 14]. In an attempt to achieve an even more effective removal of fatty acids and steroids, C18EC sorbent [5] was also investigated as sorbent, as well as Agilent's QuEChERS dSPE EMR-Lipid®, which is claimed to be especially suitable for matrices with high lipid content. Both sorbents, C18EC and Agilent's QuEChERS dSPE EMR-Lipid®, showed no significant increase of the signal areas of the pesticides compared to clean-up with MgSO4:PSA, proved with a t-test - with a significance level of 95%, and also the signal-to-noise (S/N) ratios were not improved. Consequently, these two alternative sorbents were not used in further experiments (Fig. 1).

Furthermore, the ratio of the two preferred sorbents PSA and MgSO4 was varied. Mixtures of 75 mg PSA and MgSO4 in the ratios of 1:4, 2:3, and 3:2 (m/m) were tested. Experiments showed that an increased quote of PSA has no significant effect on recovery with a probability of 95%. Therefore, we used a mixture of 75 mg PSA and MgSO4 1:4 for all following experiments.

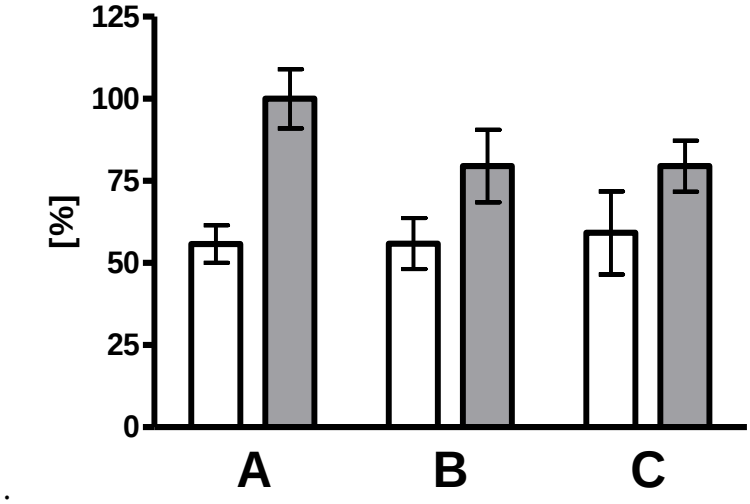

Figure 1: Evaluation of extraction solvent and dSPE sorbent. Sum of the peak areas of all 9 pesticides (1.50 mg kg^{-1}) expressed in %. **White** acetontrtile, **grey** ethyl acetate; **A** 75 mg PSA:$MgSO_4$, (1:4); **B** 75 mg PSA:C18EC:$MgSO_4$ (1:1:4), **C** 75 mg Agilent QuEChERS dSPE EMR-Lipid®; error bars show the standard error of the mean (SEM) (n = 6).

Method Validation

The validation of the novel micro-QuEChERS method was performed following SANCO/12571/2013 [12]. Every reading point was evaluated with six replicates. The selectivity was ensured by the use of a multi-reaction monitoring (MRM) working method and by the specific retention time of each analyte. No interfering peaks from endogenous compounds were found in the range of the retention times of the pesticides of interest.

The linearity of the method was assessed by generating matrix-matched curves and expressed by the coefficient of determination (r2). The calibration curves were recorded using pesticide-free blank sample matrix (fish food mix), which was spiked with pesticides in concentrations (n = 6) from 0.20 to 10.0 mg kg−1. To generate the calibration curves, the peak areas (ion count) were plotted versus the pesticide concentration. Six samples were spiked after the workup procedure with the 1.50 mg kg−1 pesticides (mean concentration of the reading points of the calibration curves) and were used to determine the precision of the instrument. The

relative standard deviations (RSD) of the instrument precision for the pesticides were between of 5 and 13%. The precision of the method was determined by the analysis of six spiked samples with 1.50 mg kg−1 of the pesticides. The relative standard deviations (RSD) of the method precision for the pesticides were between 11 and 18%. Recovery analyses were performed with pesticidefree blank sample matrix. The recoveries were calculated by comparing the measured areas of spiked samples and measured areas of the reading points of the calibration curve. The detected recoveries were between 84 and 110%. The limits of detection (LOD) and the limits of quantification (LOQ) were determined by calculating the signal-to-noise ratio of spiked samples. For the LOD a signal-to-noise ratio of 3:1, and for the LOQ a signal-to-noise ratio of 10:1 were used. The results are given in Table 1.

Application to real samples

Proof of concept was performed by analysing seven samples of animals collected from different vineyards at different time points (n = 3). The samples were potentially contaminated with Cyflufenamide, Difenoconazole and Fluopicolide (three out of nine possible pesticides), according to documented data on the application of pesticides in the respective vineyards. While no residues of Cyflufenamide and Difenoconazole could be detected, Fluopicolide was found in three samples. The contaminated samples were all picked at the same vineyard, immediately after application of the pesticides. Pooled preys of P. muralis showed, directly after pesticide exposure, a concentration of Fluopicolide of 1.68 mg kg−1. After 1 and 3 days after pesticide application, Fluopicolide could still be detected, but the measured residues are in the range between LOD and LOQ (0.15–1.00 mg kg−1). No other pesticide residues were detectable in the prey samples with the newly developed micro-QuEChERS protocol.

Conclusion

The QuEChERS technique, previously applied mainly for sample workup in the determination of pesticides in vegetables, can also be applied to trace analysis in insects and related prey of reptiles. Due to the very small amounts of biomaterial available from open land sources, a miniaturization of the QuEChERS technique had to be worked out. The novel "micro-QuEChERS" technique allows a risk assessment for endangered reptiles in intensively farmed environments.

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

Valentin Mingo[1], Stefan Lötters[1] and Norman Wagner[1]

[1]Trier University, Department of Biogeography, Universitätsring 15, 54296 Trier, Germany

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.

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.

Introduction

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

Materials and methods

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 (Figure 1). All sites have been used for viniculture for more than 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 (Figure 1). Here, wall lizards use natural rocky outcrops, rocky slopes and dry vegetation as habitat (Schulte 2008).

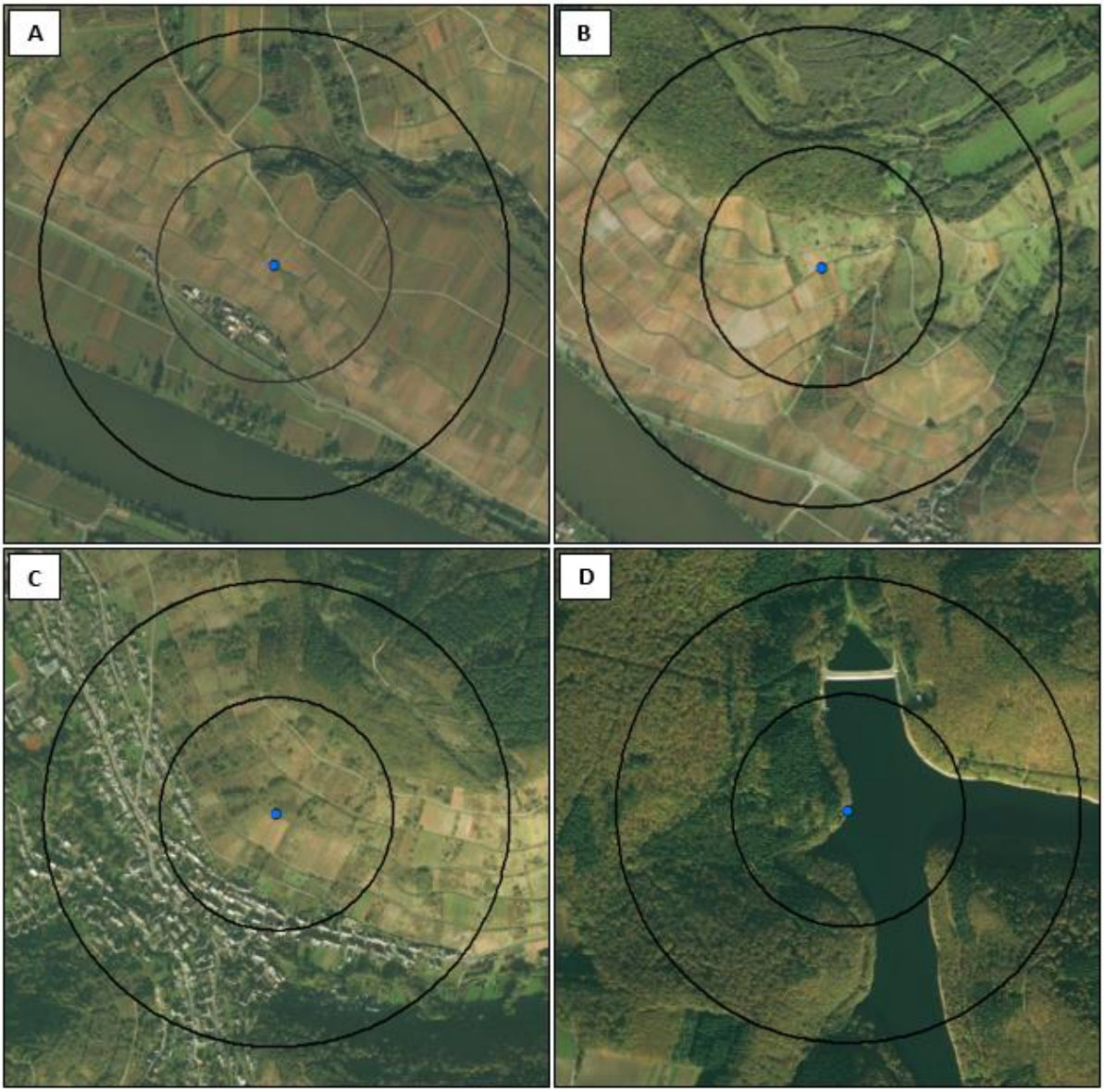

Figure 1: Surveyed sampling sites, surrounded by a 500 m (inner circle) and 1000 m (outer circle) buffer: (**A**) Lörsch – 70% agricultural land use within 1000 m; (**B**) Longen – 40% agricultural land use within 1000 m; (**C**) Fell – 10% agricultural land use within 1000 m; (**D**) Riveris – 0% agricultural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

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

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 & 400g /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	800g /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 & 37.5g/kg	Fungicide	2.4

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).

Lizard sampling

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

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).

Enzymatic assays

Frozen buccal swabs were thawed on ice and subsequently homogenized with a Mini-Beadbeater-24 homogenizer (Biospec®). Lysis buffer consisted of 25mM Tris-HCl and 0.1% Triton X-100. Samples were homogenized for 45 sec 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 µmol/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. 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 were 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 µmol/mg^{-1} protein/min, using a molar extinction coefficient of 1.36×10^4 $M^{-1}cm^{-1}$. All assays were performed at 25°C. All chemicals were obtained from Sigma-Aldrich (Munich, Germany).

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:

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

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.

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 of the area defined by *k* biomarkers arranged in a radar diagram (Beliaeff and Burgeot 2002; Figure 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.

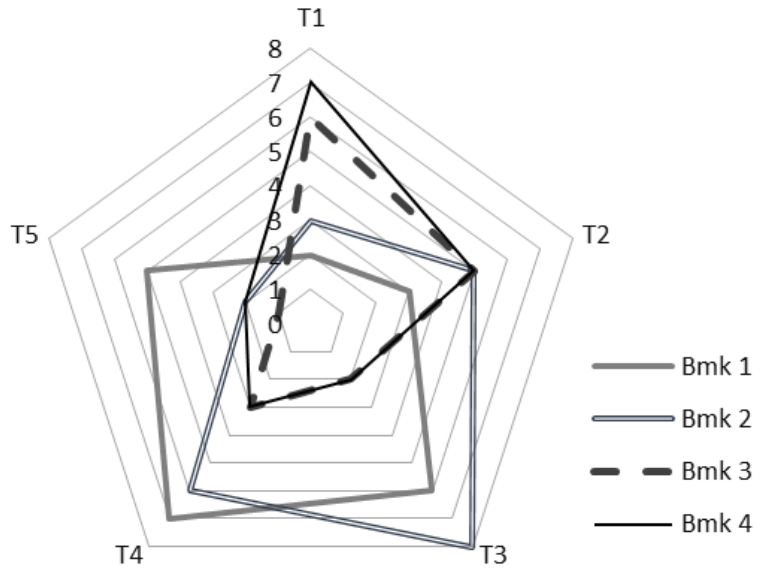

Figure 2: Example of a star plot used for the calculation of the integrated biomarker response (IBR, as defined by Beliaeff and Burgeot 2002), using four biomarkers (Bmk 1 to 4) and five time points (T1 to T5) per sampling site. Each axis of the star plot represents a standardized value (S_i) of a biomarker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

In a subsequent step, the score (S) was calculated following the equation S = Z + |Min|, where S ≥ 0 and |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:

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

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

$\beta = a\arctan\left(\frac{S_{i+1} \sin\alpha}{S_i - S_{i+1} \cos\alpha}\right)$ (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.

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).

Results

Enzymatic activity rates – fungicide applications

Enzymatic activity rates for all studied biomarkers (GST, GR, AChE) and sampling sites are summarized in Figure 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, $p > 0.05$, $df = 4$, $x^2 = 4$).

Enzymatic activity rates – herbicide application

Enzymatic activity rates regarding the studied biomarkers after exposure to Clinic Ace® had taken place are depicted in Figure 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, $p < 0.05$, $df = 2$, $x^2 = 44$).

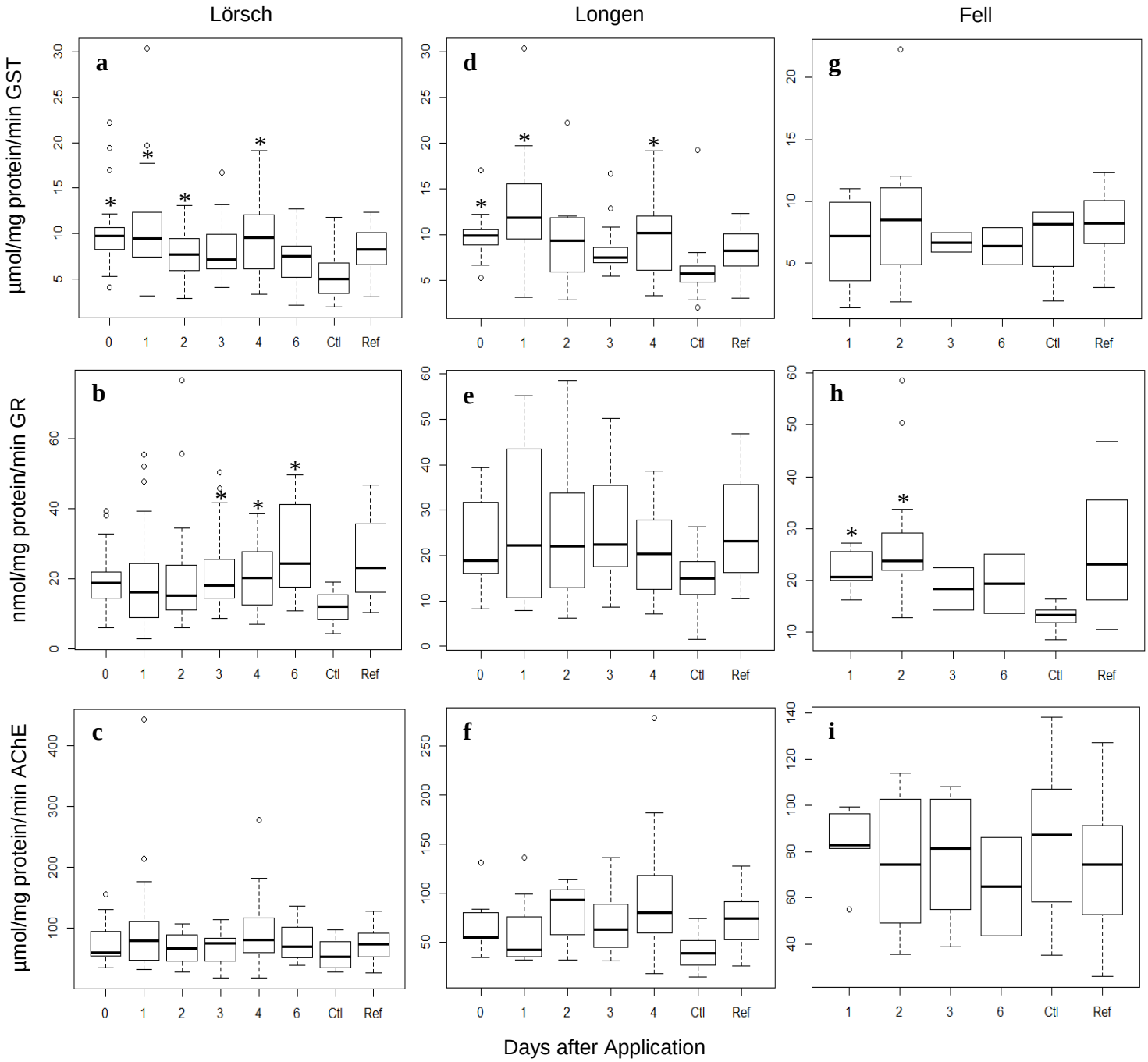

Figure 3: GST, GR and AChE activity rates for studied individuals exposed to fungicide formulations along the sampling sites: GST activity rates are depicted in sections **a**, **d** and **g**. GR activity rates are represented in sections **b**, **e** and **h**, while AChE is depicted in sections **c**, **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).

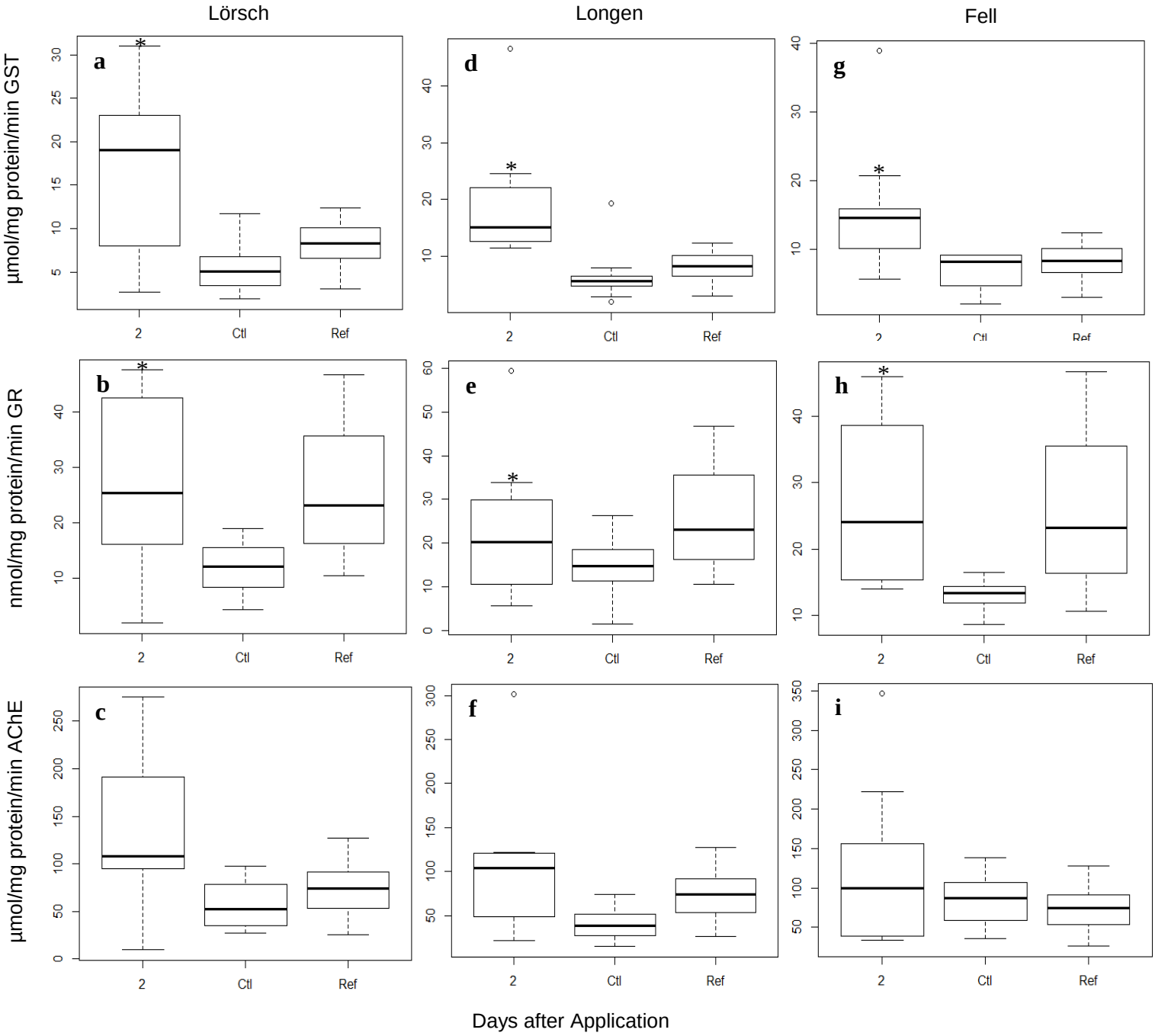

Figure 4: GST, GR and AChE activity rates for studied individuals exposed to the herbicide formulation Clinic Ace® along the sampling sites: GST activity rates are depicted in sections **a**, **d** and **g**. GR activity rates are represented in sections **b**, **e** and **h**, while AChE is depicted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

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, $p > 0.05$, $df = 2$, $x^2 = 37$).

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).

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 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 Riveris site ratio was 0.78:1.

Integrated biomarker response

IBR star plots are provided in Figure 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.

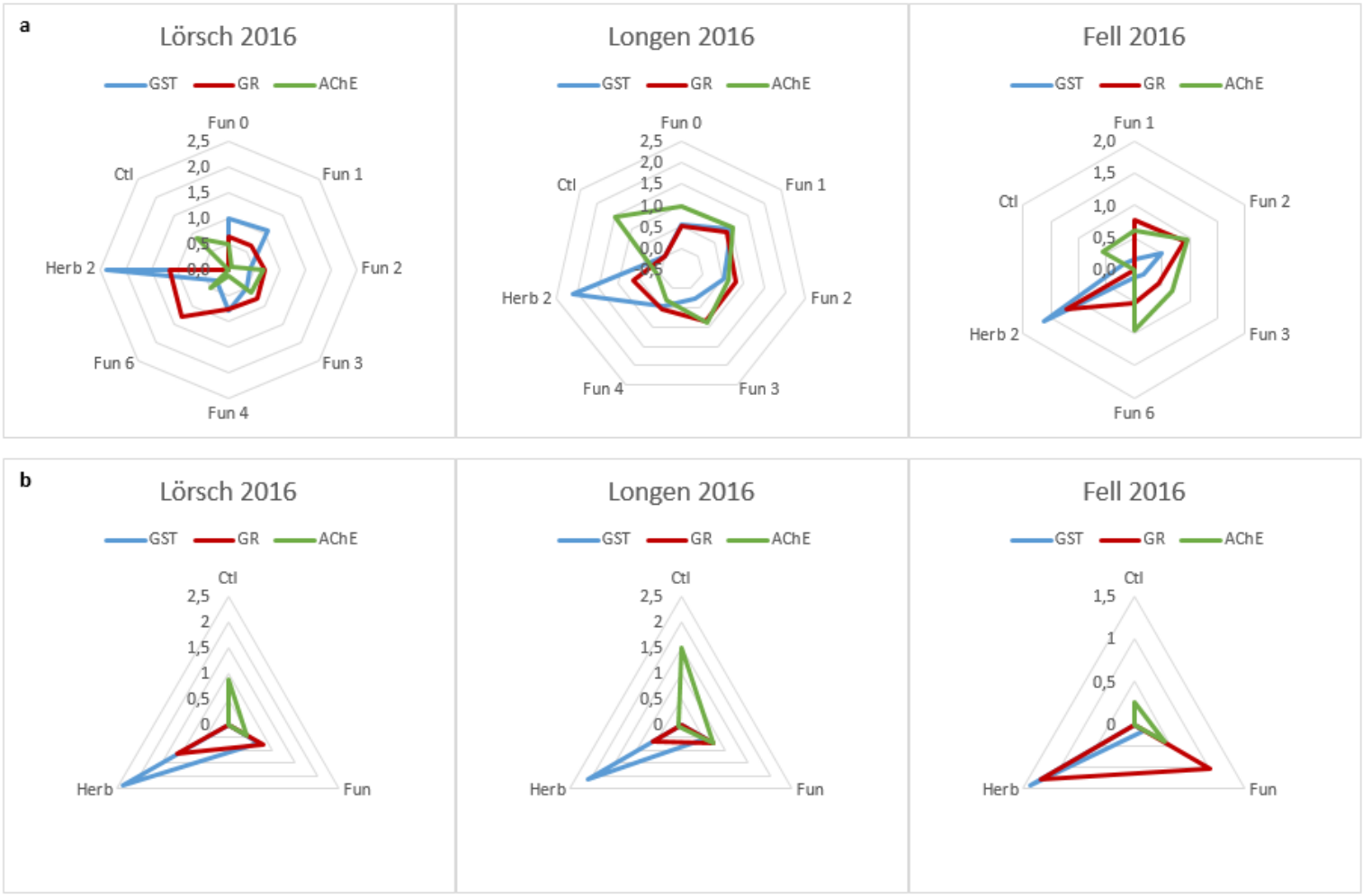

Figure 5: Star plots used to calculate the IBR for the three exposed sampling sites: Section **a** depicts specific days following a pesticide application, section **b** shows the obtained scores for reference individuals, individuals exposed to fungicides and individuals exposed to a herbicide. Legend: 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.

When observing the enzymatic activity rates during 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.

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				

Discussion

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 (Figure 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 m, 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 homoeothermic 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 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 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.

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.

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 et al. 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 López 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.

Limits of a field survey

The measured enzymatic activity rates are strong indicators toward 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.

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.

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Supplements

Supplementary material

CHAPTER I

Appendices to Wagner et al. 2015: "Risk evaluation of pesticide use to protected European reptile species"

Appendix A: "Historical" 'Land use with regular Pesticide Applications' (LPA) in 2000 and current LPA within 'Special Areas of Conservation' (SAC) that were created for Annex II reptile species and subspecies, within 1 km buffer around available presence data, 'Species Risk Index' (SRI), $SRI_{weighted}$, 'Pesticide Risk Factor' (PRF) and $PRF_{weighted}$ for each species, sorted by total size of SAC. Above-average values are written in bold, decreases/increases of %LPA in italics.

Species a	**No. of SAC**	**Area (km²) of SAC (Ø ± SE)**	**"Historical" LPA (km²) within SAC (%; Ø ± SE)**	**Current LPA within SAC ± changes (km²) (%; Ø ± SE)**	**%LPA (± SE) within buffers**	**SRI**	**SRIweighted**	**PRF**	**PRFweighted**
Emys orbicularis	1,164	151,416.38 (137.78 ± 9.89)	35,340.15 (23.34 %; 32.16 ± 2.64)	35,376.09 *+ 35.94* (**23.36 %;** 32.19 ± 2.63)	44.78 ± 3.56 (n = 100)	**14**	**2.643**	**017**	**021**
Mauremys leprosa	335	88,19713 (263.28 ± 22.66)	23,75612 (26.94 %; 70.91 ± 7.12)	23,66755 *– 88.57* (**26.84 %;** 70.65 ± 7.13)	51.75 ± 3.85 (n = 100)	**12**	**2.500**	**017**	**022**
Testudo hermanni	454	54,870.48 (120.86 ± 13.07)	11,746.10 (21.41 %; 25.87± 3.31)	12,001.04 *+ 254.94* (**21.87 %;** 26.43 ± 3.34)	22.62 ± 1.12 (n = 100)	**14**	**2.643**	**016**	**019**
Lacerta schreiberi	216	53,886.78 (249.48 ± 24.12)	8,365.95 (15.53 %; 38.73 ± 6.14)	8,350.65 *– 15.30* (**15.50 %;** 38.66 ± 6.12)	25.96 ± 2.85 (n = 100)	**13**	1.571	**011**	008
Elaphe quatuorlineata	521	42,941.41 (97.81 ± 9.99)	10,171.76 (23.69 %; 23.17 ± 3.24)	10,13236 *– 39.40* (**23.60 %;** 23.13 ± 3.25)	NA c	7	1.429	**009**	**011**
Testudo graeca	211	42,849.52 (203.08 ±	7,713.08 (18.00 %;	7,714.80 *+ 1.72*	NA c	**17**	**2.857**	**016**	**017**

		32.56)	36.55 ± 5.40)	(**18.00 %;** 37.09 ± 5.42)					
Iberolacert a (Lacerta) monticola	84	21,424.15 (255.05 ± 28.13)	1,572.34 (7.34 %; 18.72 ± 3.93)	1,562.70 *– 9.64* (7.29 %; 18.60 ± 3.88)	7.18 ± 1.58 (n = 100)	11	1.500	004	004
Vipera ursinii	42	11,346.05 (270.14 ± 111.91)	864.58 (7.62 %; 20.59 ± 11.30)	86.085 *– 3.73* (7.59 %; 20.50 ± 11.11)	27.78 ± 4.99 (n = 38)	10	**2.179**	004	006
Mauremys caspica	17	7,700.74 (452.98 ± 160.58)	2,314.34 (30.05 %; 136.14 ± 49.75)	2,312.04 *– 2.30* (**30.02 %;** 136.00 ± 50.14)	NA c	10	**2.357**	**016**	**017**
Euleptes europaea (Phyllodact ylus europaeus)	84	7,451.92 (88.71 ± 13.32)	681.88 (9.15 %; 8.12 ± 3.07)	676.64 *– 5.24* (9.08 %; 8.06 ± 3.07)	NA c	**12**	1.571	006	005
*Zamenis (Elaphe) situla*b	95	6,379.15 (67.15 ± 19.96)	1,662.60 (26.06 %; 17.50 ± 5.04)	1,766.47 *+ 103.87* (**27.69 %;** 18.59 ± 5.14)	NA c	11	**2.071**	**016**	**019**
Testudo marginataa	23	2,889.88 (125.65 ± 24.96)	470.19 (16.27%; 20.44 ± 10.52)	470.37 *+ 0.18* (**16.28 %;** 20.45 ± 10.51)	NA c	**12**	**2.321**	**010**	**013**
Iberolacerta (Lacerta) bonnali	25	2,658.13 (106.33 ± 17.19)	1.66 (0.05 %; 0.07 ± 0.04)	493 *+ 3.27* (0.19 %; 0.20 ± 0.09)	0.00 ± 0.00 (n = 25)	**13**	1.643	000	000
*Hierophis (Coluber) cypriensis**	10	993.13 (99.31 ± 58.77)	11.93 (1.20 %; 1.19 ± 0.87)	11.93 *± 0.00* (1.20 %; 1.19 ± 0.87)	NA c	10	893	001	000
*Vipera ursinii rakosiensis *d*	2	578.32 (289.16 ± 129.05)	282.47 (48.84 %; 201.84 ± 60.60)	260.96 *– 21.51* (**45.12 %;**	NA c	10	**2.179**	**024**	**033**

				130.48 ± 64.44)					
Podarcis	7	36.386	1.326	1.323	56.93 ±	8	1.286	002	002
lilfordi		(51.98 ± 27.60)	(3.64 %; 1.89 ± 0.91)	*– 0.03* (3.64 %; 1.89 ± 0.92)	7.38 (n = 26)				
Podarcis pityusensis	9	249.09 (27.68 ± 17.45)	12.23 (4.91 %; 1.36 ± 0.83)	12.81 *+ 0.58* (5.14 %; 1.43 ± 0.87)	0.00 ± 0.00 (n = 17)	11	**2.500**	003	004
Chalcides simonyi	4	202.83 (50.71 ± 25.83)	6.01 (2.96 %; 1.50 ± 0.66)	7.93 *+ 1.92* (3.91 %; 1.98 ± 0.98)	NA c	11	1.500	002	002
Gallotia galloti	2	142.82 (71.41 ±	7.99 (5.59 %;	876	NA c	**12**	1.571	004	003
insulanagae		71.31)	3.99 ± 3.99)	*+ 0.77* (6.13 %; 4.377 ± 4.377)					
*Gallotia simonyi**	4	136.16 (34.04 ± 29.98)	3.21 (2.36 %; 0.80 ± 0.80)	1.82 *– 1.39* (1.34 %; 0.46 ± 0.45)	NA c	11	1.500	001	001
*Natrix natrix cypriaca**	2	46.20 (23.10 ± 22.72)	3.16 (6.83 % ; 1.58 ± 1.51)	3.16 *± 0.00* (6.84 %; 1.58 ± 1.51)	NA c	5	536	002	001
				Ø 14.32 ± 2.61		**Ø 11.14** ± 0.56	**Ø 18.69** ± 1.35	**Ø 0.09** ± 0.02	

***** European priority species of the Habitats Directive

a The marine turtle species *Caretta caretta* and *Chelonia mydas* have not been evaluated. Excluding Greece due to the lack of land cover data; therefore the Cyclades blunt-nosed viper (*Macrovipera schweizeri*) that only occurs on the western Cyclade islands of Greece could not been evaluated.

b The Four-lined snake (*Elaphe quatuorlineata*) and the Leopard snake (*Zamenis [Elaphe] situla*) have large parts of their distributions in Greece (without actual land cover data) and Balkan countries that are not member states yet.

c NA = not sufficient occurrence data available in the databases “GBIF” and “HerpNET”

d *Vipera ursinii rakosiensis* is still listed for the Natura 2000 site “AT1220000” but already extinct in Austria. Therefore, this site was excluded.

Appendix B: 'Evaluation Factor' (EF) 1 (habitat exposure) and 2 (physiology, consisting of max. 4 RP) of the 'Species Risk Index' (SRI) and weighted to a relative scale of 0-10 (= sum of RP/0.4) for the SRIweighted

Species	**Habitats (EF 1)** (literature- based)a	**Habitats (EF 1)** (logistic regressions)b	**Relative scale for EF 1 (0-10)**	**Average snout- vent-length (EF 2)** (literature-based)	**Relative scale for EF 2 (0-10)**	**References**
Testudines c						
Common tortoise (*Testudo graeca*)	**1**	NA d	**10**	**4** (18.5 cm)	**10**	Willemsen & Hailey, 2003
Hermann's tortoise (*Testudohermanni)*	1	**1** ($z = 5.83$, $p < 0.001$)	**10**	**4** (17.7 cm)	**10**	Willemsen & Hailey, 1999; Jackson, 1980; Willemsen & Hailey, 2003
Marginated tortoise *(Testudo marginata*)	**1**	NA d	**10**	**3** (24.7 cm)	**75**	Willemsen & Hailey, 2003
European pond turtle (*Emys orbicularis*)	1	**1** ($z = 3.06$, $p < 0.05$)	**10**	**4** (12.8 cm)	**10**	Zuffi & Meozzi , 1999
Caspian turtle (*Mauremys caspica*)	**1**	NA d	**10**	**4** (16.6 cm)	**10**	Metin *et al.*, 2008
Spanish pond turtle (*Mauremys leprosa*)	1	**1** ($z = 5.04$, $p < 0.001$)	**10**	**4** (11.9 cm)	**10**	Lovich *et al.*, 2010
Sauria						
Pyrenean rock lizard (*Iberolacerta [Lacerta] bonnali*)	0	**0** ($z = 0.01$, $p > 0.05$)	**0**	**4** (5.4 cm)	**10**	Arribas & Galán, 2005
Iberian rock lizard (*Iberolacerta [Lacerta] monticola*)	0	**0** ($z = 0.34$, $p > 0.05$)	**0**	**4** (7.6 cm)	**10**	Martín & Salvador, 1993
Schreiber's green lizard (*Lacerta schreiberi*)	1	**1** ($z = 2.49$, $p < 0.05$)	**10**	**4** (9.5 cm)	**10**	Marco & Pérez-Mellado, 1999
Tenerife lizard (*Gallotia galloti insulanagae*)	**0**	NA e	**0**	**4** (16.3 cm)	**10**	Herrel *et al.*, 1999
El Hierro giant lizard (*Gallotia simonyi*)*	**0**	NA e	**0**	**4** (18.4 cm)	**10**	Rodríguez-Domínguez & Molina-Borja,

						1998
Lilford's wall lizard (*Podarcis lilfordi*)	0	**0** (z = 0.00, p > 0.05)	**0**	**4** (6.5 cm)	**10**	Ortega *et al.*, 2013
Ibiza wall lizard (*Podarcis pityusensis*)	0	**1** (z = 2.89, p < 0.01)	**10**	**4** (5.7 cm)	**10**	Carretero *et al.*, 1995
Canarian cylindrical skink (*Chalcides simonyi*)	**0**	NA d	**0**	**4** (11.4 cm)	**10**	Nogales *et al.*, 1998
European leaf-toed gecko (*Euleptes europaea [Phyllodactylus europaeus]*)	**0**	NA e	**0**	**4** (4.2 cm)	**10**	Salvidio & Oneto , 2008
Serpentes e						
Cyprus whip snake (*Hierophis [Coluber] cypriensis*)*	**0**	NA d	**0**	**1** (80.0 cm)	**25**	Zuffi *et al.*, 2007
Four-lined snake (*Elaphe quatuorlineata*)f	**1**	NA d	**10**	**0** (130.0 cm)	**0**	Filippi *et al.*, 2005
Leopard snake (*Zamenis [Elaphe] situla) f*	**1**	NA d	**10**	**2** (55.0 cm)	**5**	Zuffi & Carlino, 2010
Cyprus grass snake (*Natrix natrix cypriaca*)*	**0**	NA d	**0**	**1** (70.0 cm)	**25**	Ahmadzadeh *et al.*, 2011; Baier & Wiedl, 2010; Blosat, 2008
Meadow viper (*Vipera ursinii*) including **V. u. rakosiensis**	1	**1** (z = 3.16, p < 0.01)	**10**	**3** (37.8 cm)	**75**	Luiselli *et al.*, 2007

***** European priority species of the Habitats Directive
a For species without sufficient occurrence data for statistical analysis, the literature based Risk Points were considered (n = 11)
b Possible for only nine species
c The marine turtle species *Caretta caretta* and *Chelonia mydas*, which are European priority species, have not been evaluated.
d NA = not sufficient occurrence data available in the databases 'GBIF' and 'HerpNET'
e Excluding Greece due to the lack of land cover data; therefore, the Cyclades blunt-nosed viper (*Macrovipera schweizeri*) that only occurs on the western Cyclade islands of Greece could not been evaluated.
f The Four-lined snake (*Elaphe quatuorlineata*) and the Leopard snake (*Zamenis [Elaphe] situla*) have large parts of their distributions in Greece (without actual land cover data) and Balkan countries that are not member states yet.

Appendix C: 'Evaluation Factor' (EF) EF 3 (life history, consisting of max. 14 RP) of the 'Species Risk Index' (SRI) and weighted to a relative scale of 0-10 (= sum of RP/1.4) for the SRIweighted.

Species	**Average clutch sizes/No. of offspring** (literature-based)	**Average reproductions per year** (literature-based)	**Average age to reach sexual maturity** (literature-based)	**Relative scale for EF 3 (0-10)**	**References**
	Testudines a				
Common tortoise (*Testudo graeca*)	**4** (6.0 eggs)	**2** (2.0)	**6** (8.5 years)	**86**	Rouag *et al.*, 2007
Hermann's tortoise (*Testudo hermanni*)	**3** (7.5 eggs)	**1** (2.5)	**5** (8.0 years)	**64**	Hailey, 1989; Hailey & Willemsen, 2003
Marginated tortoise *(Testudo marginata*)	**2** (9.0 eggs)	**2** (20)	**4** (7.0 years)	**57**	Willemsen & Hailey, 2003
European pond turtle (*Emys orbicularis*)	**3** (7.0 eggs)	**3** (1.5)	**3** (6.0 years)	**64**	Zuffi *et al.*, 1999
Caspian turtle (*Mauremys caspica*)	**2** (9.0 eggs)	**3** (1.5)	**0** (3.0 years)	**36**	Auer & Taskavak, 2004
Spanish pond turtle (*Mauremys leprosa*)	**2** (8.5 eggs)	**2** (2.0)	**3** (6.0 years)	**50**	Lovich *et al.*, 2010
	Sauria				
Pyrenean rock lizard (*Iberolacerta [Lacerta] bonnali*)	**5** (4.0 eggs)	**3** (1.0)	**1** (3.5 years)	**64**	Arribas & Galán, 2005
Iberian rock lizard (*Iberolacerta [Lacerta] monticola)*	**4** (6.0 eggs)	**3** (15)	**0** (3.0 years)	**50**	Elvira & Vigal, 1985, Rúa & Galán, 2003
Schreiber's green lizard (*Lacerta schreiberi*)	**4** (6.0 eggs)	**3** (1.0)	**1** (4.0 years)	**57**	Marco *et al.*, 1994
Tenerife lizard (*Gallotia galloti insulanagae*)	**4** (6.0 eggs)	**3** (1.0)	**1** (3.5 years)	**57**	Castanet & Baez, 1991

El Hierro giant lizard (*Gallotia simonyi*)*	**3** (7.0 eggs)	**3** (1.0)	**1** (3.5 years)	**50**	Molina-Borja & Rodríguez-Domínguez, 2004
Lilford's wall lizard (*Podarcis lilfordi*)	**4** (5.0 eggs)	**0** (3.0)	**0** (2.0 years)	**29**	Ortega *et al.*, 2014
Ibiza wall lizard (*Podarcis pityusensis*)	**4** (5.0 eggs)	**3** (10)	**0** (2.0 years)	**50**	Carretero *et al.*, 1995
Canarian cylindrical skink (*Chalcides simonyi*)	**4** (4.5 eggs)	**3** (1.5)	**0** (3.0 years)	**50**	Nogales *et al.*, 1998
European leaf-toed gecko (*Euleptes europaea [Phyllodactylus europaeus]*)	**5** (2.0 eggs)	**3** (1.0)	**0** (3.0 years)	**57**	Salvidio & Delaugerre, 2003
	Serpentes b				
Cyprus whip snake (*Hierophis [Coluber] cypriensis*)*	**4** (6.0 eggs)	**3** (1.0)	**2** (4.5 years)	**64**	Zuffi *et al.*, 2007
Four-lined snake (*Elaphe quatuorlineata*)d	**1** (10.5 eggs)	**3** (10)	**2** (4.5 years)	**43**	Filippi *et al.*, 2005
Leopard snake (*Zamenis [Elaphe] situla*)d	**4** (5.0 eggs)	**3** (1.0)	**1** (4.0 years)	**57**	Moravec & Böhme, 2005
Cyprus grass snake (*Natrix natrix cypriaca*)*	**0** (16.0 eggs)	**3** (1.0)	**1** (4.0 years)	**29**	Luiselli & Capula, 2007; Blosat, 2008
Meadow viper (*Vipera ursinii*) including *V. u. rakosiensis**	**3** (7.0 neonates) e	**3** (1.0)	**0** (3.0 years)	**43**	Lyet *et al.*, 2009; Zamfirescu *et al.*, 2009

***** European priority species of the Habitats Directive
a The marine turtle species *Caretta caretta* and *Chelonia mydas*, which are European priority species, have not been evaluated.
b Excluding Greece due to the lack of land cover data; therefore, the Cyclades blunt-nosed viper (*Macrovipera schweizeri*) that only occurs on the western Cyclade islands of Greece could not been evaluated.
c according to *Hierophis viridiflavus*
d The Four-lined snake (*Elaphe quatuorlineata*) and the Leopard snake (*Zamenis [Elaphe] situla*) have large parts of their distributions in Greece (without actual land cover data) and Balkan countries that are not member states yet
e Whole species considered

Appendix D: Differences in 'Land use with regular Pesticide Applications' (%LPA) within national 'Special Areas of Conservation' (SAC) that were created for Annex II reptile species, which occur in more than one EU member state (n = 12), sorted after total size of SAC. Statistically significant national differences are written in bold.

Species, national differences a	**Country**	**No. of national SAC**	**Total area (km²) of national SAC (Ø ± SE)**	**LPA (km²) within SAC (%; Ø ± SE)**
Emys orbicularis $F = 1.02$, $df = 1$, $p > 0.05$	Austria	5	432.72 (86.54 ± 25.81)	96.45 (22.29 %; 19.29 ± 7.09)
	Bulgaria	189	30,958.12 (163.80 ± 27.03)	6,608.87 (21.35 %; 34.97 ± 5.50)
	Germany	13	566.35 (43.57 ± 16.42)	73.50 (12.98 %; 5.65 ± 2.10)
	Spain	136	46,837.62 (344.39 ± 45.08)	10,677.81 (22.80 %; 78.51 ± 12.87)
	France	115	12,874.03 (111.95 ± 32.57)	2,614.50 (20.31 %; 22.73 ± 9.54)
	Hungary	168	12,166.64 (72.42 ± 10.16)	2,803.32 (23.04 %; 16.69 ± 2.92)
	Italy	368	19,176.43 (52.11 ± 6.04)	5,246.55 (27.36 %; 14.26 ± 2.10)
	Lithuania	3	179.16 (59.72 ± 55.32)	71.25 (39.77 %; 23.75 ± 22.83)
	(Latvia) b	(1)	(3.825)	(4.51 (11.80 %))
	Poland	28	5,370.15 (191.79 ± 52.43)	833.36 (15.52 %; 29.76 ± 10.12)
	Portugal	15	10,147.74 (676.52 ± 86.47)	3,815.50 (37.60 %; 254.37 ± 52.45)
	Romania	39	11,889.03 (304.85 ± 119.00)	2,125.97 (17.88 %; 54.51 ± 14.05)
	Slovenia	14	338.18 (24.16 ± 10.21)	167.93 (49.66 %; 12.00 ± 6.60)
	Slovakia	5	441.96 (88.39 ± 65.51)	236.57 (53.53 %; 47.31 ± 41.86)
Mauremys leprosa $F = 5.78$, $df = 1$, $\mathbf{p < 0.05}$	Spain	290	69,966.46 (241.26 ± 24.84)	17,109.31 (24.45 %; 59.00 ± 7.18)
	(France) b	(1)	(6.957)	(8.98 (12.91 %))

	Portugal	44	18,161.09 (412.75 ± 49.09)	6,549.26 (36.06 %; 148.85 ± 23.56)
Lacerta schreiberi $F = 14.28$, $df = 1$, $\boldsymbol{p < 0.001}$	Spain	186	43,051.23 (231.46 ± 25.31)	5,492.31 (12.76 %; 29.53 ± 5.24)
	Portugal	30	10,835.55 (361.19 ± 72.33)	2,858.34 (26.38 %; 95.28 ± 28.00)
Elaphe quatuorlineata $F = 9.02$, $df = 1$, $\boldsymbol{p < 0.01}$	Bulgaria	144	20,309.76 (141.04 ± 25.18)	5,089.46 (25.06 %; 35.34 ± 6.51)
	Italy	288	21,317.49 (74.02 ± 10.24)	4,860.41 (22.80 %; 16.94 ± 3.68)
	Romania	6	1,262.77 (210.46 ± 131.24)	164.64 (13.04 %; 27.44 ± 10.66)
	(Slovenia) b	(1)	(5.138)	(17.85; 35 %)
Testudo hermanni $F = 8.81$, $df = 1$, $\boldsymbol{p < 0.01}$	Bulgaria	178	32,398.70 (181.45 ± 28.92)	6,405.26 (19.83 %; 35.99 ± 5.81)
	Spain	29	1,762.98 (60.79 ± 19.68)	269.54 (15.30 %; 9.30 ± 2.23)
	France	32	1,728.57 (54.02 ± 19.89)	97.31 (5.63 %; 3.04 ± 1.82)
	Italy	208	16,321.36 (78.47 ± 10.81)	4,890.18 (29.96 %; 23.51 ± 5.20)
	Romania	7	2,758.88 (394.13 ± 169.91)	338.74 (12.28 %; 48.39 ± 16.67)
Testudo graeca $F = 13.48$, $df = 1$, $\boldsymbol{p < 0.001}$	Bulgaria	153	31,484.40 (205.78 ± 33.18)	6,354 (20.18 %; 41.53 ± 6.67)
	Spain	25	2,945.04 (117.80 ± 47.07)	318.76 (10.82 %; 13.28 ± 4.44)
	Italy	21	2,335.05 (111.19 ± 25.67)	335.11 (14.35 %; 16.76 ± 8.45)
	Romania	12	6,085.03 (507.09 ± 372.32)	706.75 (11.62 %; 64.25 ± 38.16)
Iberolacerta (Lacerta) monticola	(Spain)	(83)	(20,541.36 (247.49 ± 27.26))	(1,424.13 (6.93 %; 17.16 ± 3.62))
	(Portugal) b	(1)	(88.279)	(138.57 (15.70 %))
Vipera ursinii $F = 7.20$, $df = 1$, $\boldsymbol{p < 0.05}$	France	11	732.25 (73.26 ± 16.05)	12.53 (1.71 %; 1.25 ± 0.71)
	Hungary	4	518.60 (129.65 ± 6.31)	71.20 (13.73 %; 17.80 ± 5.37)

	Italy	24	5,537.15 (230.72 ± 69.07)	309.24 (5.59%; 12.89 ± 5.39)
	Romania	4	4,558.05 (1,139.51 ± 1,132.13)	467.88 (10.26 %; 116.97 ± 113.17)
Mauremys caspica $F = 3.80$, $df = 1$, $p > 0.05$	Bulgaria	14	6,250.93 (446.50 ± 175.79)	1,734.31 (27.75 %; 123.88 ± 49.99)
	Cyprus	2	5.10 (2.55 ± 1.55)	4.42 (86.67 %; 2.21 ± 1.46)
	(Spain) b	(1)	(1,44477)	(573.31 (39.68 %))
Euleptes europaea (Phyllodactylus europaeus) $F = 4.68$, $df = 1$, $\mathbf{p < 0.05}$	France	24	2,415.12 (100.63 ± 34.30)	15.05 (0.62 %; 0.63 ± 0.20)
	Italy	60	5,036.80 (83.95 ± 12.83)	661.60 (13.14 %; 11.03 ± 4.24)
Zamenis (Elaphe) situla $F = 3.71$, $df = 1$, $p > 0.05$	Bulgaria	11	3,571.64 (324.69 ± 137.84)	496.04 (13.89 %; 45.09 ± 19.53)
	Italy	77	2,777.55 (36.07 ± 10.42)	1,264.17 (45.51 %; 16.42 ± 5.60)
	Malta	7	29.96 (4.28 ± 3.16)	6.27 (20.92 %; 0.90 ± 0.52)
Lacerta (Iberolacerta) bonnali $F = 0.16$, $df = 1$, $p > 0.05$	Spain	8	1,290.31 (161.29 ± 41.20)	1.28 (0.10 %; 0.16 ± 0.13)
	France	17	1,367.83 (80.46 ± 13.11)	3.65 (0.27 %; 0.22 ± 0.12)

a Differences between group means of LPA proportions in national SAC were checked using one-way ANOVA; some data had to be Box-Cox transformed prior analysis. The Four-lined snake (*Elaphe quatuorlineata*) and the Leopard snake (*Zamenis [Elaphe] situla*) have large parts of their distributions in Greece (without actual land cover data) and Balkan countries that are not member states yet. The marine turtle species *Caretta caretta* and *Chelonia mydas* have not been evaluated. Excluding Greece due to the lack of land cover data; therefore the Cyclades blunt-nosed viper (*Macrovipera schweizeri*) that only occurs on the western Cyclade islands of Greece could not been evaluated.
b not part of analysis

Appendix E: Contingency table that contains the results of the Bonferroni-corrected post-hoc-tests for *Elaphe quatuorlineata*, *Testudo hermanni*, *T. graeca*, *Vipera ursinii*, and. Post-hoc-tests for other species did not show significant differences between member states. Abbreviations: n.s. = not significant; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; AT = Austria; BE = Belgium; BG = Bulgaria; CZ = Czech Republic; DE = Germany; DK = Denmark; EE = Estonia; FI = Finland; FR = France; HU = Hungary; IT = Italy; LU = Luxembourg; LT = Lithuania; LV = Latvia; NL = Netherlands; PL = Poland; RO = Romania; SE = Sweden; SI = Slovenia; SK = Slovakia.

Mauremys leprosa

	ES	**PT**
ES		*
PT	*	

Lacerta schreiberi

	ES	**PT**	
ES		***	
PT	***		

Elaphe quatuorlineata

	BG	**IT**	**RO**
BG		*	n.s.
IT	*		n.s.
RO	n.s.	n.s.	

Testudo hermanni

	BG	**ES**	**FR**	**IT**	**RO**
BG		n.s.	***	n.s.	n.s.
ES	n.s.		n.s.	n.s.	n.s.
FR	***	n.s.		**	n.s.
IT	n.s.	n.s.	**		n.s.
RO	n.s.	n.s.	n.s.	n.s.	

Testudo graeca

	BG	**ES**	**IT**	**RO**
BG		***	*	n.s.
ES	***		n.s.	n.s.
IT	*	n.s.		n.s.
RO	n.s.	n.s.	n.s.	

Vipera ursinii

	FR	HU	IT	RO
FR		n.s.	n.s.	***
HU	n.s.		n.s.	**
IT	n.s.	n.s.		***
RO	***	**	***	

Euleptes europaea (Phyllodactylus europaeus)

	FR	IT
FR		n.s.
IT	n.s.	

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Appendix to Mingo et al. 2016: "Risk of pesticide exposure for reptile species in the European Union"

Data on life history, physiological traits and occurrence within agricultural areas for all reviewed species. RP = Risk Points, SVL = Snout-to-vent length, BM = Body mass, ARA = Agricultural areas with regular pesticide applications, ERI = Exposure Risk Index, ERF = Exposure Risk Factor, CR = Critically Endangered, EN = Endangered, VU = Vulnerable, NT = Near Threatened, LC = Least Concern, DD = Data Deficient.

Species	RP Habitat	Clutch size	RP	N° of Clutches	RP	BM (g)	RP	SVL (cm)	RP	RP Physiology	RP Life history	%ARA	ERI	ERF	Red List Category	Source
Ablepharus kitaibelii	0	3	7	1	5	1,5	11	3,7	8	10,00	10,00	71	20,00	**0,47**	LC	Budak et al. 1998; Herczeg et al.. 2007; Meiri. 2010; Glandt. 2010; Szövényi & Jelíc. 2011
Acanthodactylus erythrurus	0	4,4	6	1,5	5	8,7	11	6,8	8	10,00	9,17	44	19,17	**0,28**	LC	Böhme. 1981; Belliure. 2009; Meiri. 2010
Algyroides fitzingeri	1	3	7	1	5	2	11	3	8	10,00	10,00	29	30,00	**0,29**	LC	Böhme. 1981; Capula et al.. 2002
Algyroides marchi	0	2,5	7	2	4	2,5	11	5	8	10,00	9,17	11	19,17	**0,07**	EN	Böhme. 1981; Fernández-Cardenete & García-Cardenete. 2010
Algyroides nigropunctatus	1	2	7	2	4	3	11	6	8	10,00	9,17	20	29,17	**0,19**	LC	Böhme. 1981; Arnold. 1987; Bressi. 2004
Anguis fragilis	1	6,7	5	1,5	5	13,5	10	17	7	8,95	8,33	49	27,28	**0,45**	LC	Böhme. 1981; Ferreiro & Galán. 2004; Sahlean et al.. 2008; Galán & Salvador. 2009; Salvador. 2009; Meiri. 2010
Archaeolacerta bedriagae	0	4,5	6	1,5	5	9,3	11	7,1	8	10,00	9,17	12	19,17	**0,08**	NT	Meiri. 2010; Glandt. 2010
Chalcides bedriagai	1	3	7	1	5	6,7	11	8,2	8	10,00	10,00	52	30,00	**0,52**	NT	Böhme. 1981; Martín & Lopez. 2002; Pollo. 2009; Meiri. 2010; Glandt. 2010
Chalcides chalcides	0	6,5	5	1	5	6,3	11	12,7	7	9,47	8,33	74	17,81	**0,44**	LC	Böhme. 1981; Almeida & Almeida. 1986; Rugiero. 1997; Glandt. 2010; Meiri. 2010
Chalcides coeruleopunctatus	0	3,5	6	1	5	6,6	11	8	8	10,00	9,17	24	19,17	**0,15**	LC	Salvador. 2008; Glandt. 2010

Chalcides ocellatus	1	7	5	1,5	5	27	9	12,4	7	8,42	8,33	53	26,75	**0,47**	NT	Daut & Andrews, 1993; Böhme. 1981; Rabou et al. 2007; Meiri. 2010
Chalcides sexlineatus	0	2,5	7	1	5	7,8	11	11	7	9,47	10,00	26	19,47	**0,17**	LC	Brown, 1992; Salvador & Brown. 2009a; Meiri. 2010; Glandt. 2010
Chalcides simonyi	0	4,5	6	1,5	5	24,3	9	11,4	7	8,42	9,17	19	17,59	**0,11**	EN	Nogales et al.. 1998; Salvador. 2009a; Glandt. 2010
Chalcides striatus	0	5	6	1	5	6,8	11	12	7	9,47	9,17	69	18,64	**0,43**	LC	Ceacero et al.. 2007; Meiri. 2010; Glandt. 2010; Pollo. 2012
Chalcides viridanus	0	3,5	6	1	5	6,6	11	8,1	8	10,00	9,17	18	19,17	**0,11**	LC	Salvador. 2009b; Sánchez-Hernández et al.. 2013
Chamaeleo chamaeleon	1	23	1	1	5	40,8	8	10,3	7	7,89	5,00	47	22,89	**0,36**	LC	Böhme. 1981; Dimaki et al.. 2000; Hódar et al.. 2000; Cuadrado et al., 2003; Cuadrado. 2009; Meiri. 2010; Cattaneo. 2012
Coronella austriaca	1	8,3	5	2	4	45	8	40,9	5	6,84	7,50	60	24,34	**0,49**	LC	Santos et al.. 2009; Galán. 2009a; Glandt. 2010; Feldman & Meiri. 2013
Coronella girondica	1	6	6	1	5	55	8	37,5	6	7,37	9,17	25	26,54	**0,22**	LC	Feriche et al.. 1993; Santos & Pleguezuelos. 2009; Glandt. 2010; Feldman & Meiri. 2013
Darevskia praticola	0	5	6	1	5	2,5	11	5	8	10,00	9,17	56	19,17	**0,36**	NT	Sahlean et al.. 2008; Glandt. 2010; Meiri. 2010
Dinarolacerta mosorensis	0	4	6	1	5	5,8	11	6,3	8	10,00	9,17	17	19,17	**0,11**	VU	Glandt. 2010; Meiri. 2010
Dolichopis caspius	1	11	4	1	5	600	1	110	2	1,58	7,50	48	19,08	**0,31**	LC	Huyghe et al.. 2007; Krčmar et al.. 2007; Cattaneo. 2008; Glandt. 2010; Covaciu-Marcov et al.. 2012; Cattaneo & Cattaneo. 2014
Dolichopis jugularis	0	10	4	1	5	691	1	100	3	2,11	7,50	43	9,61	**0,14**	LC	Göcmen et al.. 2008a; Glandt. 2010; Feldman & Meiri. 2013
Elaphe quatuorlineata	0	10,5	4	1	5	700	1	130	1	1,05	7,50	75	8,55	**0,21**	NT	Luiselli & Rugiero. 1990; Cattaneo. 2005; Filippi et al.. 2005 ; Feldman & Meiri. 2013
Elaphe sauromates	0	10	4	1	5	800	1	110	2	1,58	7,50	73	9,08	**0,22**	LC	Feldman & Meiri. 2013; Cattaneo & Cattaneo. 2014
Emys orbicularis	1	7	5	1,5	5	355	3	12,8	7	5,26	8,33	52	23,60	**0,41**	NT	Zuffi et al.. 1999; Auer & Taskavak. 2004; Ayres. 2009

Emys trinacris	0	12,5	3	1,5	5	337	3	12	7	5,26	6,67	65	11,93	**0,26**	DD	D'Angelo et al.. 2008; Glandt. 2010
Eremias arguta	0	4	6	1,5	5	11,3	10	6,1	8	9,47	9,17	75	18,64	**0,47**	NT	Böhme. 1981; Meiri. 2010
Eryx jaculus	0	13	3	1	5	31	9	55	5	7,37	6,67	14	14,04	**0,07**	LC	Buttle. 1989; Rabou et al.. 2007; Ghergel et al.. 2009; Glandt. 2010; Feldman & Meiri. 2013; Cattaneo & Cattaneo. 2014
Euleptes europaea	0	2	7	1	5	1,5	11	4,2	8	10,00	10,00	24	20,00	**0,16**	NT	Böhme. 1981; Salvidio & Delaugerre. 2003; Salvidio & Oneto. 2008
Gallotia atlantica	0	2,5	7	1,5	5	12,8	10	7,5	8	9,47	10,00	54	19,47	**0,35**	LC	Baez & Castanet. 1991 ; Molina-Borja & Rodríguez-Domínguez. 2004; Salvador. 2009; Meiri. 2010; Lopez-Darias et al.. 2015
Gallotia caesaris	0	3	7	1,5	5	10	11	6,5	8	10,00	10,00	20	20,00	**0,13**	LC	Baez & Castanet. 1991 ; Molina-Borja & Rodríguez-Domínguez. 2004; Salvador. 2009c; Lopez Darias et al.. 2015
Gallotia galloti	0	4,5	6	1	5	40	9	9,5	8	8,95	9,17	49	18,11	**0,30**	LC	Baez & Castanet. 1991; Molina-Borja & Rodríguez-Domínguez. 2004; Salvador. 2009e; Fariña et al.. 2011; Meiri. 2010; Lopez-Darias et al.. 2015
Gallotia simonyi	0	8,6	5	1,5	5	295	4	14,8	7	5,79	8,33	11	14,12	**0,05**	CR	Rodríguez-Domínguez & Molina-Borja. 1998; Molina-Borja & Rodríguez-Domínguez. 2004; Meiri. 2010; Salvador. 2014; Lopez-Darias et al.. 2015
Gallotia stehlini	1	9,8	4	1	5	208	4	16,8	7	5,79	7,50	85	23,29	**0,66**	LC	Baez & Castanet. 1991 ; Molina-Borja & Rodríguez-Domínguez. 2004; Salvador. 2009f; Meiri. 2010; Lopez-Darias et al.. 2015
Hemidactylus turcicus	1	2	7	1,5	5	3	11	5	8	10,00	10,00	52	30,00	**0,52**	LC	Böhme. 1981; Budak et al.. 1998; Bader et al.. 2009; Meiri. 2010; Lisicic et al.. 2012; Rato. 2012
Hemorrhois hippocrepis	1	7,5	5	1	5	300	4	74	4	4,21	8,33	40	22,54	**0,30**	LC	Almeida & Almeida. 1986; Feriche et al.. 1993; Feriche.

																2009; Glandt. 2010; Feldman & Meiri. 2013
Hierophis gemonensis	0	7	5	1	5	110	5	49	5	5,26	8,33	30	13,60	**0,14**	LC	Glandt. 2010
Hierophis viridiflavus	0	10	4	1	5	150	5	79	4	4,74	7,50	28	12,24	**0,11**	LC	Fornasiero et al.. 2007; Santos et al.. 2010; Feldman & Meiri. 2013
Iberolacerta aranica	0	2,5	7	1	5	2,6	11	5,3	8	10,00	10,00	1	20,00	**0,01**	EN	Arribas & Galán. 2005; Arribas. 2009a; Meiri. 2010
Iberolacerta aurelioi	0	2	7	1	5	2,7	11	5,4	8	10,00	10,00	0	20,00	**0,00**	EN	Arribas & Galán. 2005; Arribas. 2009b
Iberolacerta bonnali	0	6,2	5	1,5	5	2,8	11	5,4	8	10,00	8,33	3	18,33	**0,02**	NT	Arribas & Galán. 2005; Arribas. 2009c; Meiri. 2010
Iberolacerta cyreni	0	6	6	1	5	7,3	11	7	8	10,00	9,17	5	19,17	**0,03**	EN	Meiri. 2010; Martín. 2009a; Glandt. 2010
Iberolacerta horvathi	0	4	6	2	4	4,1	11	5,5	8	10,00	8,33	7	18,33	**0,04**	NT	Glandt. 2010
Iberolacerta monticola	0	6	6	1,5	5	7,5	11	6,5	8	10,00	9,17	17	19,17	**0,11**	VU	Elvira & Vigal. 1985; Martín & Salvador. 1993; Rúa & Galán. 2003; Martín. 2009; Meiri. 2010
Lacerta agilis	1	9,5	4	1	5	8,3	11	7,5	8	10,00	7,50	43	27,50	**0,39**	LC	Amat. 2008; Ekner et al.. 2008; Gland 2010t; Majláthová et al.. 2010; Meiri. 2010
Lacerta bilineata	1	14	3	1,5	5	30	9	10,6	7	8,42	6,67	44	25,09	**0,37**	LC	Glandt. 2010; Meiri. 2010; Sacchi et al.. 2011; Gosá & Rubio. 2013
Lacerta schreiberi	0	14	3	1	5	23,2	9	9,5	8	8,95	6,67	26	15,61	**0,14**	NT	Marco et al.. 1994; Glandt. 2010; Meiri. 2010; Marco. 2015
Lacerta trilineata	0	8	5	1,5	5	79,4	7	15	7	7,37	8,33	20	15,70	**0,10**	LC	Pafilis & Valakos. 2008; Meiri. 2010; Glandt. 2010
Lacerta viridis	0	12	4	1,5	5	39	9	12	7	8,42	7,50	65	15,92	**0,34**	LC	Strugariu et al.. 2009; Glandt. 2010; Meiri. 2010
Laudakia stellio	0	10	4	1	5	83,7	6	24,7	6	6,32	7,50	45	13,82	**0,21**	LC	Yildirimhan et al. 2006; Meiri. 2010; Glandt. 2010 Göcmen et al.. 2008b
Macroprotodon brevis	1	4,2	6	1	5	18,3	10	28	6	8,42	9,17	21	27,59	**0,19**	NT	Pleguezuelos. 2005; Glandt. 2010
Macroprotodon cucullatus	1	3	7	1	5	16,6	10	33,5	6	8,42	10,00	35	28,42	**0,33**	LC	Almeida & Almeida. 1986; Feriche et al..

																1993; Pleguezuelos. 2009a; Glandt. 2010; Feldman & Meiri. 2013
Malpolon insignitus	0	13	3	1	5	345	3	85	3	3,16	6,67	24	9,82	**0,08**	LC	Glandt. 2010; Cattaneo & Cattaneo. 2014
Malpolon monspessulanus	1	7,5	5	1	5	1257	1	84,3	3	2,11	8,33	55	20,44	**0,37**	LC	Almeida & Almeida. 1986; Feriche et al.. 2008; Bologna et al. 2006; Pleguezuelos. 2009; Feldman & Meiri. 2013
Mauremys leprosa	1	8,5	5	2	4	700	1	11,9	7	4,21	7,50	41	21,71	**0,30**	VU	Martín. 2010; Buenetxea et al.. 2010; Lovich et al.. 2010
Mauremys rivulata	1	8	5	1,5	5	487	1	15,7	7	4,21	8,33	7	22,54	**0,05**	LC	Auer & Taskavak. 2004; Metin et al. 2008
Mediodactylus kotschyi	1	1,5	7	1	5	3	11	5	8	10,00	10,00	58	30,00	**0,58**	LC	Böhme. 1981; Budak et al.. 1997; Scillitani et al.. 2004; Slavenko et al.. 2015
Montivipera xanthina	1	8	5	1	5	340	3	94	3	3,16	8,33	10	21,49	**0,07**	LC	Cattaneo. 2008; Cattaneo. 2012; Feldman & Meiri. 2013
Natrix maura	0	17	2	1	5	44,4	8	36,5	6	7,37	5,83	7	13,20	**0,03**	LC	Santos. 2009; Glandt. 2010; Feldman & Meiri. 2013
Natrix natrix	0	16	2	1	5	13	10	55	5	7,89	5,83	51	13,73	**0,23**	LC	Feriche et al.. 1993; Pleguezuelos. 2010; Ahmadzadeh et al.. 2011; Feldman & Meiri. 2013; Cattaneo & Cattaneo. 2014
Natrix tessellata	0	15	3	1	5	79,5	7	69	4	5,79	6,67	20	12,46	**0,08**	LC	Zimmermann & Fachbach. 1996; Feriche et al.. 1993; Duda et al.. 2007; Luiselli et al.. 2007; Feldman & Meiri. 2013
Ophisops elegans	0	4	6	1,5	5	3	11	4,3	8	10,00	9,17	30	19,17	**0,19**	LC	Göcmen et al.. 2008; Glandt. 2010; Meiri. 2010
Platyceps collaris	0	4	6	1	5	63,6	7	27	6	6,84	9,17	2	16,01	**0,01**	LC	Glandt. 2010; Cattaneo & Cattaneo. 2014; Feldman & Meiri. 2013
Platyceps najadum	0	4	6	1	5	60	8	57,1	5	6,84	9,17	44	16,01	**0,24**	LC	Glandt. 2010; Cattaneo & Cattaneo. 2014
Podarcis bocagei	1	3	7	3	3	4,6	11	5,4	8	10,00	8,33	28	28,33	**0,26**	LC	Galán. 2009b; Meiri. 2010; Glandt. 2010; Kaliontzopoulou et al.. 2010; Amaral et al.. 2012a,b; Bicho et al.. 2013
Podarcis carbonelli	0	3	7	1	5	3,4	11	4,9	8	10,00	10,00	24	20,00	**0,16**	EN	Sá-Sousa. 2009; Meiri. 2010; Glandt. 2010

Podarcis erhardii	0	2,5	7	1	5	9,5	11	5,9	8	10,00	10,00	32	20,00	**0,21**	LC	Herkt. 2007; Glandt. 2010; Meiri. 2010
Podarcis hispanicus	1	2,8	7	2	4	4	11	5,5	8	10,00	9,17	38	29,17	**0,37**	LC	Galán & Lannoo. 2003; Sampedro et al.. 2008; Meiri. 2010; Glandt. 2010
Podarcis lilfordi	0	2,5	7	2	4	6,9	11	6,2	8	10,00	9,17	16	19,17	**0,10**	EN	Salvador. 2009g; Meiri. 2010; Glandt. 2010; Ortega et al. 2014
Podarcis melisellensis	1	4,5	6	5	2	6	11	6	8	10,00	6,67	23	26,67	**0,20**	LC	Brecko et al.. 2008; Huyghe et al.. 2007; Meiri. 2010; Glandt. 2010; Stamenkovic & Matic. 2013
Podarcis muralis	1	5	6	2	4	6	11	5,7	8	10,00	8,33	46	28,33	**0,43**	LC	Bender et al.. 1996; Schulte. 2008; Diego-Rasilla. 2009; Meiri. 2010; Glandt. 2010
Podarcis pityusensis	0	3	7	1	5	6,8	11	6,5	8	10,00	10,00	45	20,00	**0,30**	NT	Carretero et al.. 1995; Salvador. 2009h; Meiri. 2010; Glandt. 2010
Podarcis siculus	1	7	5	3	3	6,7	11	6,8	8	10,00	6,67	58	26,67	**0,51**	LC	Biaggini et al.. 2009; Meiri. 2010; Glandt. 2010; Rivera et al.. 2011; Salvador. 2015; Stamenkovic & Matic. 2013
Podarcis tiliguerta	0	9	5	1	5	4,6	11	5,6	8	10,00	8,33	29	18,33	**0,18**	LC	Meiri. 2010; Glandt. 2010
Podarcis vaucheri	0	3	7	1	5	3,1	11	6	8	10,00	10,00	47	20,00	**0,31**	LC	Salvador & Busack. 2009; Glandt. 2010
Psammodromus algirus	1	4	6	1,5	5	9,1	11	7	8	10,00	9,17	7	29,17	**0,07**	LC	Böhme. 1981; Meiri. 2010; Salvador. 2011a
Psammodromus edwardsianus	0	3,5	6	1,5	5	1,7	11	14	7	9,47	9,17	30	18,64	**0,18**	LC	Glandt. 2010; Fitze. 2012
Psammodromus hispanicus	1	2,5	7	2	4	2,7	11	4,2	8	10,00	9,17	42	29,17	**0,41**	LC	Böhme. 1981; Meiri. 2010; Fitze. 2012
Pseudopus apodus	1	9	5	1	5	27,5	9	40	5	7,37	8,33	18	25,70	**0,15**	LC	Böhme. 1981; Huyghe et al.. 2007; Meiri. 2010; Lisicic et al.. 2012
Rhinechis scalaris	1	15	3	1	5	500	2	72	4	3,16	6,67	3	19,82	**0,02**	LC	Pleguezuelos. 2006; Glandt. 2010; Feldman & Meiri. 2013
Tarentola angustimentalis	0	1,5	7	2	4	6	11	6	8	10,00	9,17	16	19,17	**0,10**	LC	Salvador. 2009i; Glandt. 2010; García-Muñoz et al.. 2013
Tarentola boettgeri	0	1,5	7	1,5	5	6	11	5,5	8	10,00	10,00	17	20,00	**0,11**	LC	Salvador & Brown. 2009b; Glandt. 2010
Tarentola	0	1,5	7	1	5	15	10	6,8	8	9,47	10,00	20	19,47	**0,13**	LC	Salvador. 2009j; Glandt. 2010

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Tarentola gomerensis	0	1	7	6	1	6	11	5,8	8	10,00	6,67	13	16,67	**0,07**	LC	Salvador. 2009k; Glandt. 2010
Tarentola mauritanica	1	1,5	7	2	4	8,2	11	8,6	8	10,00	9,17	46	29,17	**0,45**	LC	Böhme. 1981; Valakos & Mylonas. 1992; Meiri. 2010; Salvador. 2011b; Lisicic et al.. 2012
Teira dugesii	1	2,6	7	2,5	4	9	11	6,7	8	10,00	9,17	46	29,17	**0,45**	LC	Malkmus. 1995; Molina-Borja & Rodríguez-Domínguez. 2004; Meiri. 2010
Telescopus fallax	1	7	5	1	5	50	8	13	7	7,89	8,33	30	26,23	**0,26**	LC	Kirchner. 2009; Cattaneo. 2010; Glandt. 2010; Feldman & Meiri. 2013
Testudo graeca	1	3,5	6	2,5	4	782	1	18,5	7	4,21	8,33	74	22,54	**0,56**	VU	Jackson. 1980 ; Willemsen & Hailey. 2003; Abd Rabou et al.. 2007; Guzman et al.. 2007; Rouag et al.. 2007; Arakelyan & Parham. 2008; Díaz-Paniagua & Andreu. 2009
Testudo hermanni	1	8	5	2,5	4	653	1	17,7	7	4,21	7,50	52	21,71	**0,38**	NT	Jackson. 1980; Hailey. 1990; Willemsen & Hailey. 1999; Willemsen & Hailey. 2001; Willemsen & Hailey. 2003; Bertolero. 2010; Couturier et al.. 2014
Testudo marginata	1	9	5	2	4	2094	1	24,7	6	3,68	7,50	24	21,18	**0,17**	LC	Willemsen & Hailey. 2003; Glandt. 2010
Timon lepidus	1	16	2	1	5	215	4	17	7	5,79	5,83	40	21,62	**0,29**	NT	Meiri. 2010; Glandt. 2010; Grillet et al. 2010; Mateo. 2011
Typhlops vermicularis	1	6	6	1	5	2,3	11	23,5	6	8,95	9,17	43	28,11	**0,40**	LC	Glandt. 2010; Feldman & Meiri. 2013; Cattaneo & Cattaneo. 2014
Vipera ammodytes	0	5,6	6	1	5	211	4	60,5	4	4,21	9,17	47	13,38	**0,21**	LC	Luiselli & Zuffi. 2002; Feldman & Meiri. 2013
Vipera aspis	0	6,5	5	1	5	100	6	55	5	5,79	8,33	28	14,12	**0,13**	LC	Luiselli & Zuffi. 2002; Martínez-Freiría. 2009; Feldman & Meiri. 2013
Vipera berus	0	12,5	3	1	5	74,5	7	60	5	6,32	6,67	33	12,98	**0,14**	LC	Madsen et al.. 1993; Feldman & Meiri. 2013
Vipera latastei	0	9,3	4	1,5	5	87	6	40	5	5,79	7,50	9	13,29	**0,04**	VU	Brito & Rebelo. 2003; Brito.

																2015; Feldman & Meiri. 2013
Vipera seoanei	0	6	6	1	5	106	5	50	5	5,26	9,17	11	14,43	**0,05**	LC	Brito. 2009; Glandt. 2010
Vipera ursinii	0	7	5	1	5	90	6	37,8	6	6,32	8,33	1	14,65	**0,00**	VU	Péchy et al. 2000; Újvári et al.. 2002; Luiselli et al.. 2007; Zamfirescu et al.. 2009; Feldman & Meiri. 2013
Zamenis lineatus	0	8	5	1	5	190	5	84,3	3	4,21	8,33	56	12,54	**0,23**	DD	Luiselli et al.. 2006; Glandt. 2010; Feldman & Meiri. 2013
Zamenis longissimus	0	7	5	1	5	200	5	109	2	3,68	8,33	29	12,02	**0,11**	LC	DüŞen et al.. 2010; Rubio & Gosá. 2010; Feldman & Meiri. 2013
Zamenis situla	1	5	6	1	5	350	3	55	5	4,21	9,17	31	23,38	**0,24**	LC	Buttle. 1989; Moravec & Böhme. 2003; Zuffi & Carlino. 2004; Cattaneo. 2012
Zootoca vivipara	0	7	5	2	4	4,8	11	5,5	8	10,00	7,50	70	17,50	**0,41**	LC	Ekner et al.. 2008; Glandt. 2010; Meiri. 2010; Majláthová et al.. 2010

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Supplementary material

CHAPTER II

Appendix to Mingo et al. 2017a: "The use of buccal swabs as a minimal-invasive method for detecting effects of pesticide exposure on enzymatic activity in common wall lizards"

Data on the applied pesticide formulations, their application dates and application rates, sampling dates and number of sampled individuals per day, in all sites studied during the year 2015.

Application date	Pesticide formulation	Application rate (kg/ha)	Sampling site	Sampling dates	Days after last application	Number of sampled individuals
			Lörsch	24.03.2015	0 (reference)	10
			Lörsch	08.04.2015	0 (reference)	10
15.04.2015	Touchdown®	2	Lörsch			
			Lörsch	16.04.2015	1	7
			Lörsch	20.04.2015	5	5
16.05.2015	Polyram WG®	0.8	Lörsch			
			Lörsch	18.05.2015	2	10
26.05.2015	Polyram WG® + Vivando®	2	Lörsch	19.05.2015	3	10
05.06.2015	Mildicut + Collis	3.12 + 0.5	Lörsch			
			Lörsch	08.06.2015	3	10
			Lörsch	11.06.2015	6	7
15.06.2015	Pergado® + Dynali®	2.5 + 0.63	Lörsch			
17.06.2015	Enervin® + Luna Experience®	2.5 + 0.3	Lörsch	17.06.15†	2	10
25.06.2015	Profiler® + Luna Experience®	2.81 + 0.47	Lörsch			
06.07.2015	Vento Power® + Forum Star®	2.4 + 2	Lörsch			
11.07.2015	Folpan 80 WDG® + Luna Experience®	1.6 + 0.5	Lörsch			
			Lörsch	14.07.2015	3	10
			Lörsch	17.07.2015	6	10

07.08.2015	Folpan 80 WDG® + Systhane 20 EW®	1.6 + 0.24	Lörsch			
						Total = 99
			Longen	20.03.2015	0 (reference)	10
			Longen	23.03.2015	0 (reference)	10
15.04.2015	Touchdown®	2	Longen			
			Longen	17.04.2015	2	7
16.05.2015	Polyram WG®	0.8	Longen			
26.05.2015	Polyram WG® + Vivando®	2	Longen			
05.06.2015	Mildicut® + Collis®	3.12 + 0.5	Longen			
15.06.2015	Pergado® + Dynali®	2.5 + 0.63	Longen			
17.06.2015	Enervin® + Luna Experience®	2.5 + 0.3	Longen			
25.06.2015	Profiler® + Luna Experience®	2.81 + 0.47	Longen			
06.07.2015	Vento Power® + Forum Star®	2.4 + 2	Longen			
11.07.2015	Folpan 80 WDG® + Luna Experience®	1.6 + 0.5	Longen			
			Longen	15.07.2015	4	10
			Longen	18.07.2015	7	9
07.08.2015	Folpan 80 WDG® + Systhane 20 EW®	1.6 + 0.24	Longen			
			Longen	11.08.2015	4	10
			Longen	14.08.2015	7	10
						Total = 66
			Fell	26.03.2015	0 (reference)	10
			Fell	09.04.2015	0 (reference)	10
15.04.2015	Touchdown®	2	Fell			
			Fell	22.04.2015	7	5
16.05.2015	Polyram WG®	0.8	Fell			
			Fell	20.05.2015	5	7

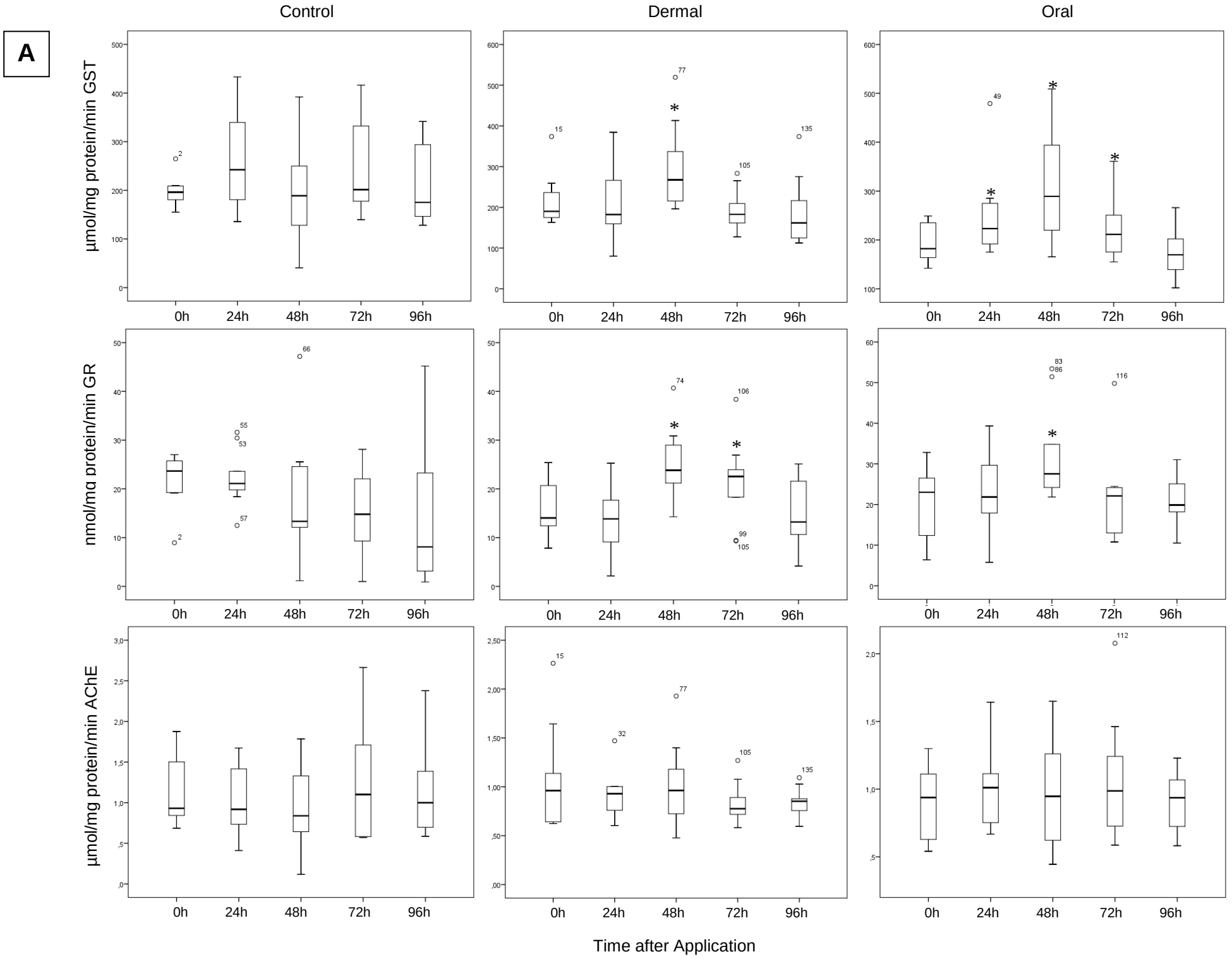
27.05.2015	Polyram WG® + Vivando®	2 + 0.2	Fell			
04.06.2015	Vivando® + Polyram WG®	0.16 + 1.16	Fell			
			Fell	05.06.2015	1	6
08.06.2015	Profiler® + Dynali®	1.88 + 0.5	Fell			
11.06.2015	Vivando® + Polyram WG®	0.16 + 2	Fell			
			Fell	16.06.2015	5	10
18.06.2015	Pergado® + Luna Experience®	2.5 + 0.39	Fell			
29.06.2015	Enervin® + Vivando®	5 + 0.4	Fell			
14.07.2015	Folpan 80 WDG® + Vento Power® + Teldor®	1.6 + 1.6	Fell			
			Fell	15.07.2015	1	5
19.07.2015	Mildicut® + Collis®	5 + 0.8	Fell			
20.07.2015	Pergado® + Dynali®	4 + 1	Fell			
			Fell	21.07.2015	1	10
30.07.2015	Folpan® + Veriphos® + Topas®	2 + 5 +0.4	Fell			
			Fell	31.07.2015	1	7
07.08.2015	Folpan 80 WDG® + Systhane 20 EW®	1.6 + 0.24	Fell			
			Fell	11.08.2015	4	10
						Total = 80

Additionally to the mentioned pesticide formulations, sulfur was applied in all sampling sites at each application date

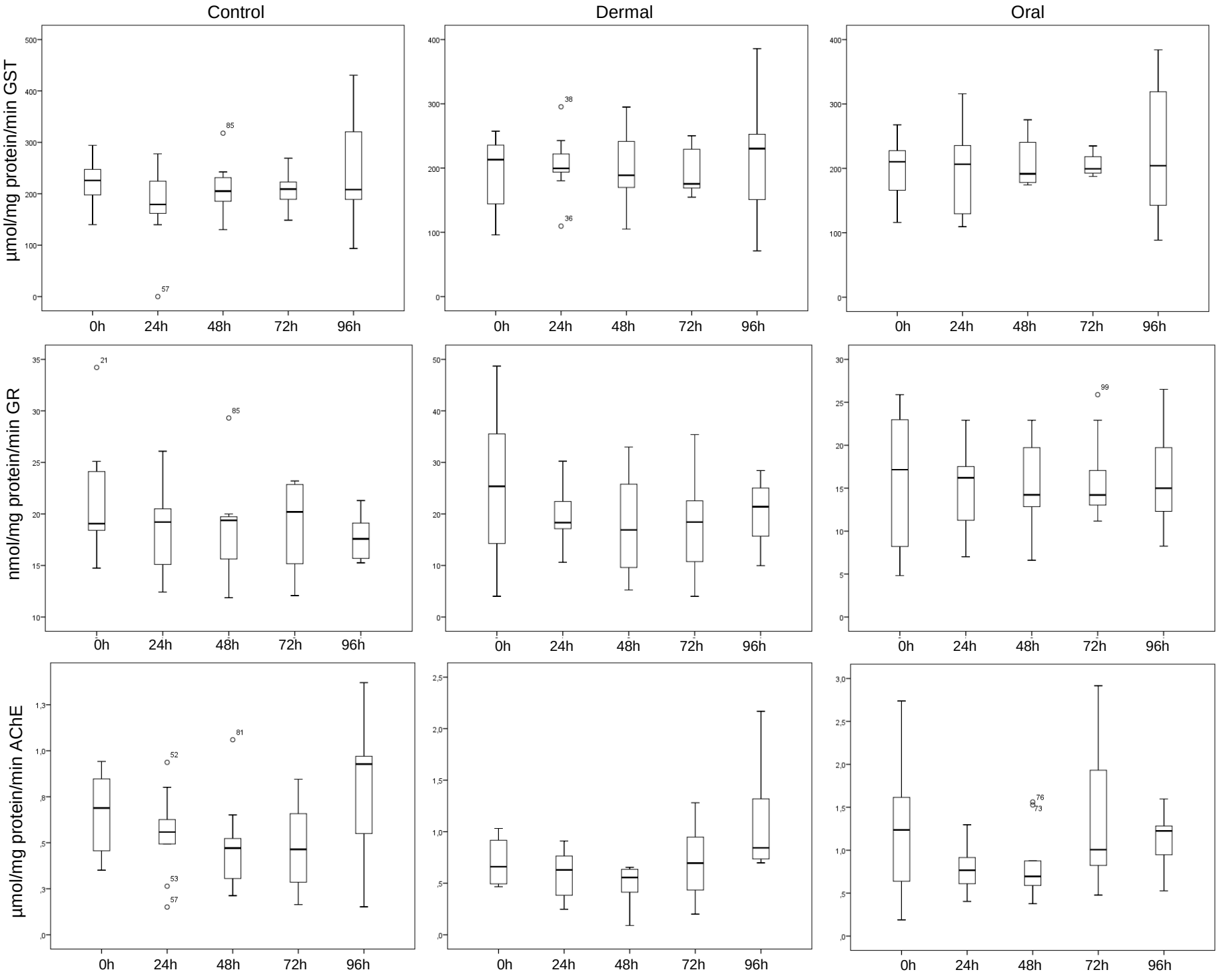
† Sampling took place before the application on 17.06.15

Appendix to Mingo et al. (*under review*): "Validating buccal swabbing as a minimal-invasive method to detect pesticide exposure in reptiles"

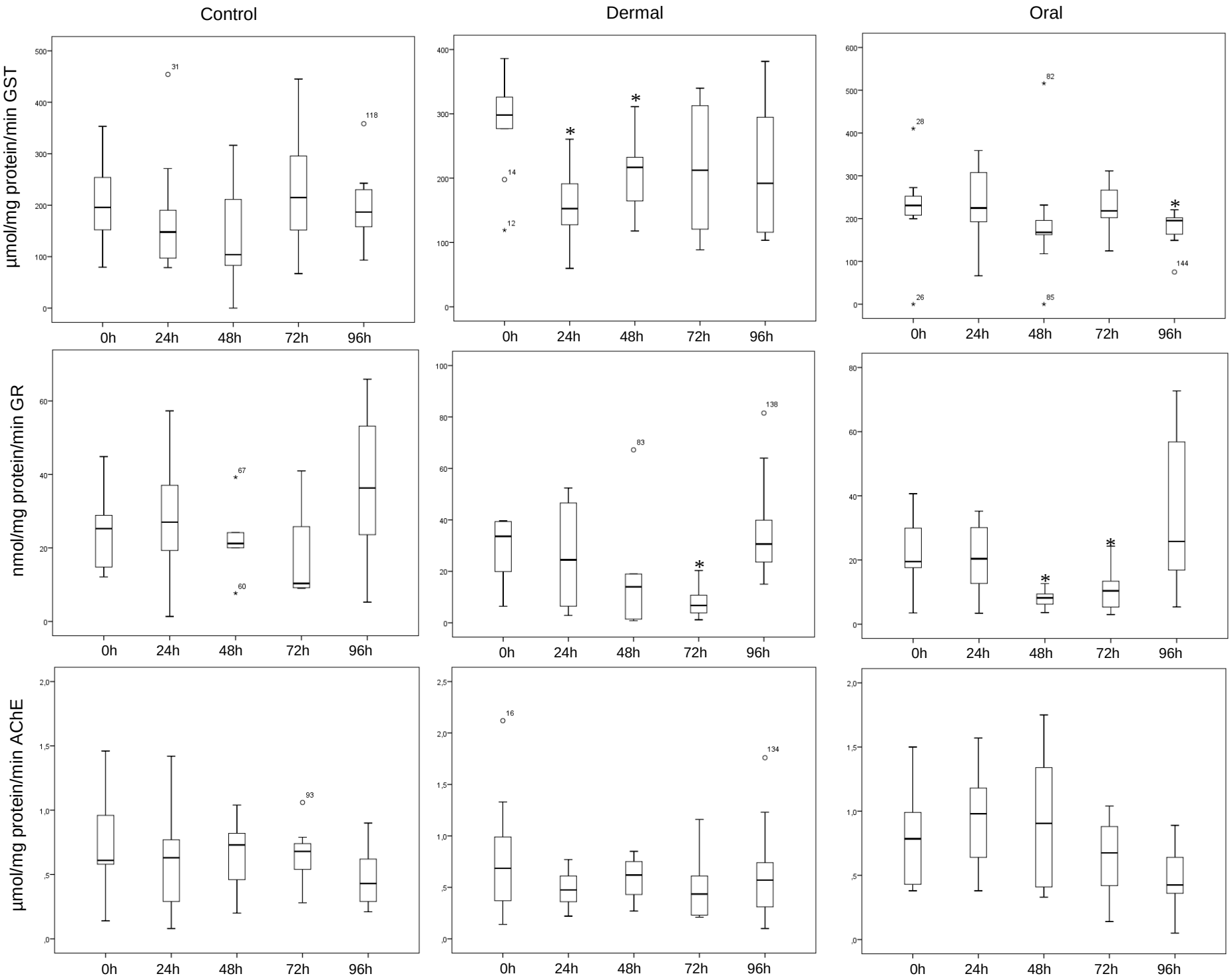
Enzymatic activities of Common wall lizards according to treatment group (control, dermal exposure, oral exposure) after exposure to the tested pesticide ormulations during a time period of 96h. GST: Glutathione-S-Transferase; GR: Glutathione reductase; AChE: Acetylcholinesterase. A: Enzymatic activities after exposure to the fungicide mix containing the formulations Enervin® and Vivando®. B: Enzymatic activities after exposure to to the single fungicide formulation Vivando®. C: Enzymatic activities after exposure to the herbicide formulation Roundup® UltraMax. * = significant differences to 0 h (pre exposure).


A
Control
Dermal
Oral
µmol/mg protein/min GST
nmol/mg protein/min GR
µmol/mg protein/min AChE
0h
24h
48h
72h
96h
Time after Application

B


Control
Dermal
Oral
µmol/mg protein/min GST
nmol/mg protein/min GR
µmol/mg protein/min AChE
0h
24h
48h
72h
96h
Time after Application

C


Control
Dermal
Oral
µmol/mg protein/min GST
nmol/mg protein/min GR
µmol/mg protein/min AChE
0h
24h
48h
72h
96h
Time after Application

Supplementary material

CHAPTER III

Appendices to Mingo et al. 2017b: "The impact of land use intensity and associated pesticide applications on fitness and enzymatic activity in reptiles — A field study"

Appendix 1: Data on the applied pesticide formulations, their application dates and application rates, sampling dates and number of sampled individuals per day, in all sampling sites studied during the year 2016.

Application date	Pesticide formulation	Application rate (kg, L/ha)	Sampling site	Sampling dates	Days after last application	Number of sampled individuals
			Lörsch	11.04.2016	Control samples	10
17.04.2016	Clinic Ace®	5	Lörsch	19.04.2016	2	7
31.05.2016	Polyram WG® + Vivando®†	2 + 0.2	Lörsch	01.06.2016	1	7
			Lörsch	02.06.2016	2	5
			Lörsch	04.06.2016	4	10
			Lörsch	06.06.2016	6	10
09.06.2016	Profiler® + Dynali®†	1.88 + 0.5	Lörsch	09.06.2016	0	10
			Lörsch	10.06.2016	1	10
20.06.2016	Enervin® + Vivando® + Vegas®+ Electis®†	3.13 + 0.25 + 0.3 + 1.8	Lörsch	20.06.2016	0	10
			Lörsch	22.06.2016	2	10
			Lörsch	23.06.2016	3	10
			Lörsch	24.06.2016	4	8
29.06.2016	Pergado® + Luna Experience®†	3 + 0.47	Lörsch			
07.07.2016	Forum Star® + Dynali®	1.44 + 0.6	Lörsch			
09.07.2016	Enervin® + Vivando®†	3.75 + 0.3	Lörsch			
19.07.2016	Mildicut® + Collis®†	5 + 0.8	Lörsch	20.07.2016	1	10
			Lörsch	21.07.2016	2	10

			Lörsch	22.07.2016	3	10
			Lörsch	25.07.2016	6	10
28.07.2016	Collis® + Fantic F®†	0.64 + 2.4	Lörsch			
16.08.2016	Folpan®	1.6	Lörsch			
						Total = 147
			Longen	14.04.2016	Control samples	10
			Longen			
18.04.2016	Clinic Ace®†	5	Longen	20.04.2016	2	10
31.05.2016	Polyram WG® + Vivando®†	2 + 0.2	Longen	31.05.2016	0	10
			Longen			
			Longen			
			Longen			
09.06.2016	Profiler® + Dynali®†	1.88 + 0.5	Longen	10.06.2016	1	8
			Longen	11.06.2016	2	7
20.06.2016	Enervin® + Vivando®†	3.13 + 0.25	Longen	21.06.2016	1	8
			Longen	22.06.2016	2	8
			Longen	23.06.2016	3	10
			Longen			
29.06.2016	Pergado® + Luna Experience®†	3 + 0.47	Longen			
07.07.2016	Forum Star® + Dynali®	1.44 + 0.6	Longen			
09.07.2016	Enervin® + Vivando®†	3.75 + 0.3	Longen	12.07.2016	3	10
			Longen	13.07.2016	4	10
19.07.2016	Mildicut® + Collis®†	5 + 0.8	Longen			
28.07.2016	Collis® + Fantic F®†	0.64 + 2.4	Longen			
16.08.2016	Folpan®	1.6	Longen			
						Total = 91
			Fell	12.04.2016	Control samples	10
			Fell			

19.04.2016	Clinic Ace®	5	Fell	21.04.2016	2	9
01.06.2016	Polyram WG® + Vivando®†	2 + 2.2	Fell	03.06.2016	2	7
			Fell	07.06.2016	6	10
10.06.2016	Profiler® + Dynali®†	2 + 0.5	Fell	11.06.2016	1	7
			Fell			
21.06.2016	Pergado® + Electis® + Luna Experience®†	2.5 + 1.8 + 0.39	Fell	22.06.2016	1	10
			Fell	23.06.2016	2	10
28.06.2016	Enervin® + Vivando®†	3 + 0.24	Fell			
30.06.2016	Enervin® + Vivando®†	3.75 + 0.3	Fell			
			Fell			
10.07.2016	Mildicut® + Collis®	5 + 0.8	Fell	13.07.2016	3	10
			Fell			
21.07.2016	Pergado® + Vento Power®	4 + 2	Fell	23.07.2016	2	7
28.07.2016	Collis® + Fantic F®†	0.64 + 2.4	Fell			
30.07.2016	Teldor®	1.6	Fell			
16.08.2016	Folpan®	1.6	Fell			
						Total = 80
			Riveris	15.06.2016		10
			Riveris	17.08.2016		11
			Riveris	06.09.2016		10
			Riveris	07.09.2016		10
						Total = 41

† Additionally to the mentioned pesticide formulations, sulfur was applied in the sampling site

Appendix 2: Condition indices and biometric data collected from wall lizards sampled along an agricultural gradient during the years 2015 and 2016.

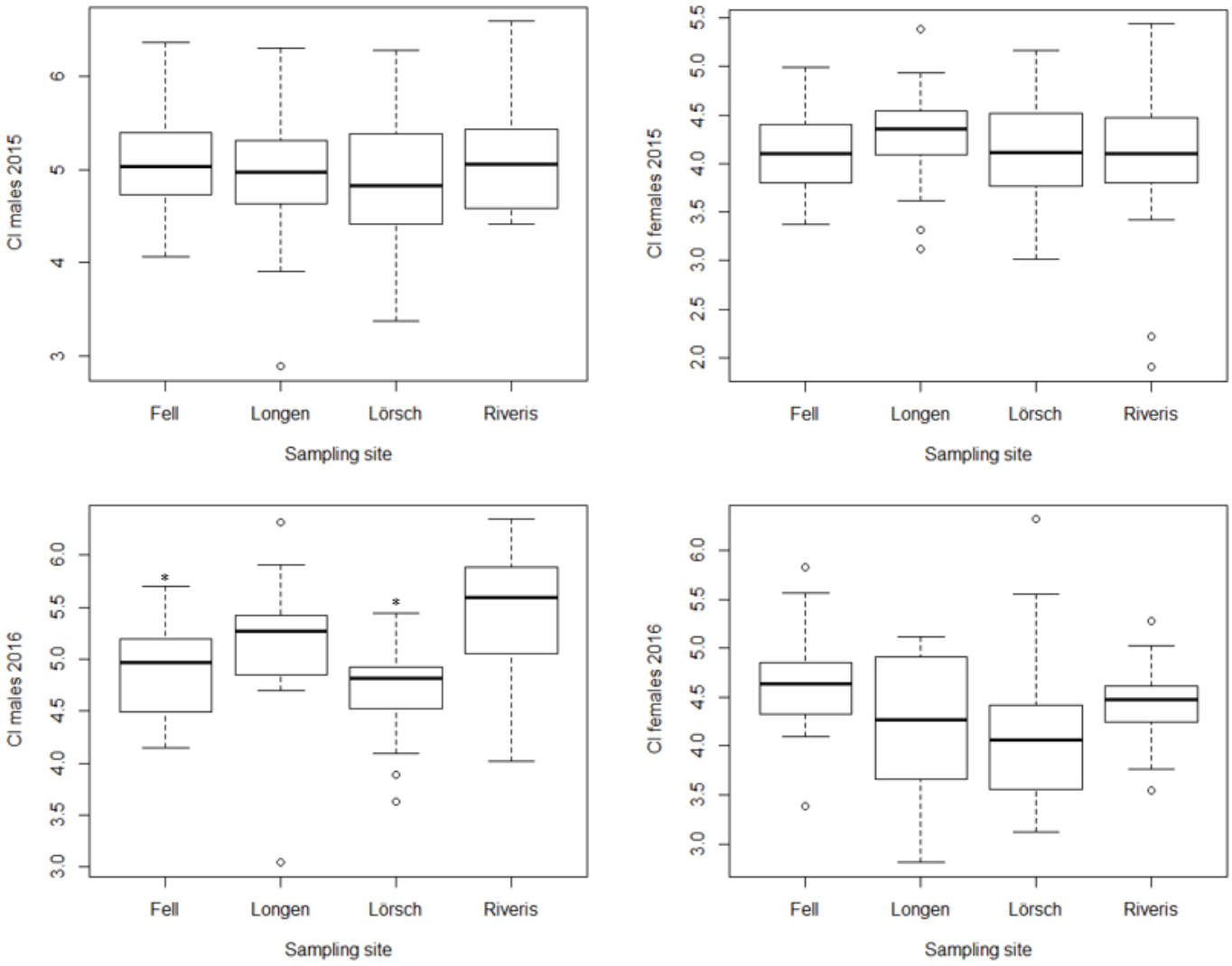


Fig. 1: Condition indices (CI) of male and female wall lizards sampled along an agricultural gradient during the years 2015 and 2016. * = significant difference compared to the reference site (Riveris).

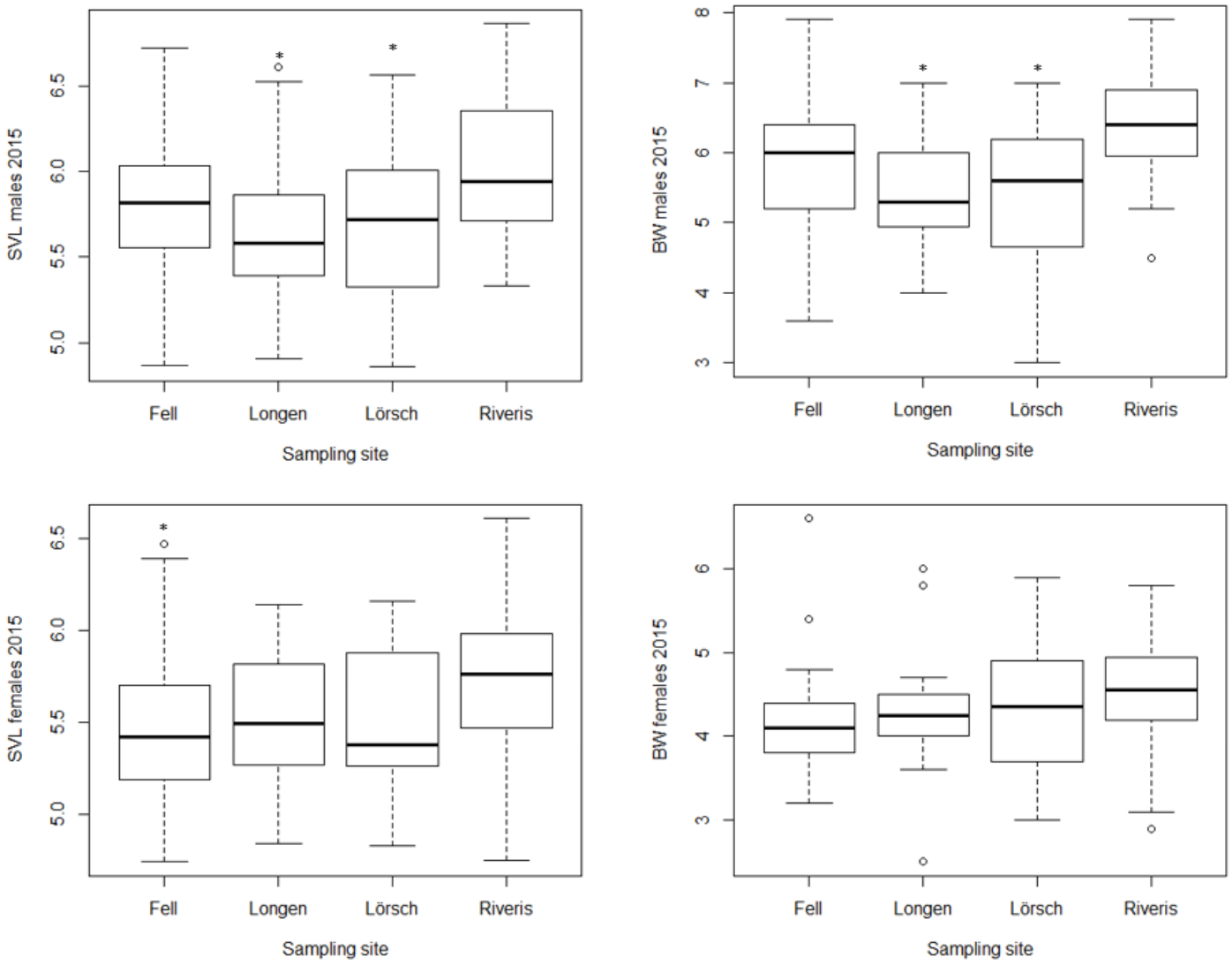


Fig. 2: Snout-to-vent-length (cm) and body mass (g) of male and female wall lizards sampled along an agricultural gradient during the year 2015. * = significant difference compared to the reference site (Riveris).

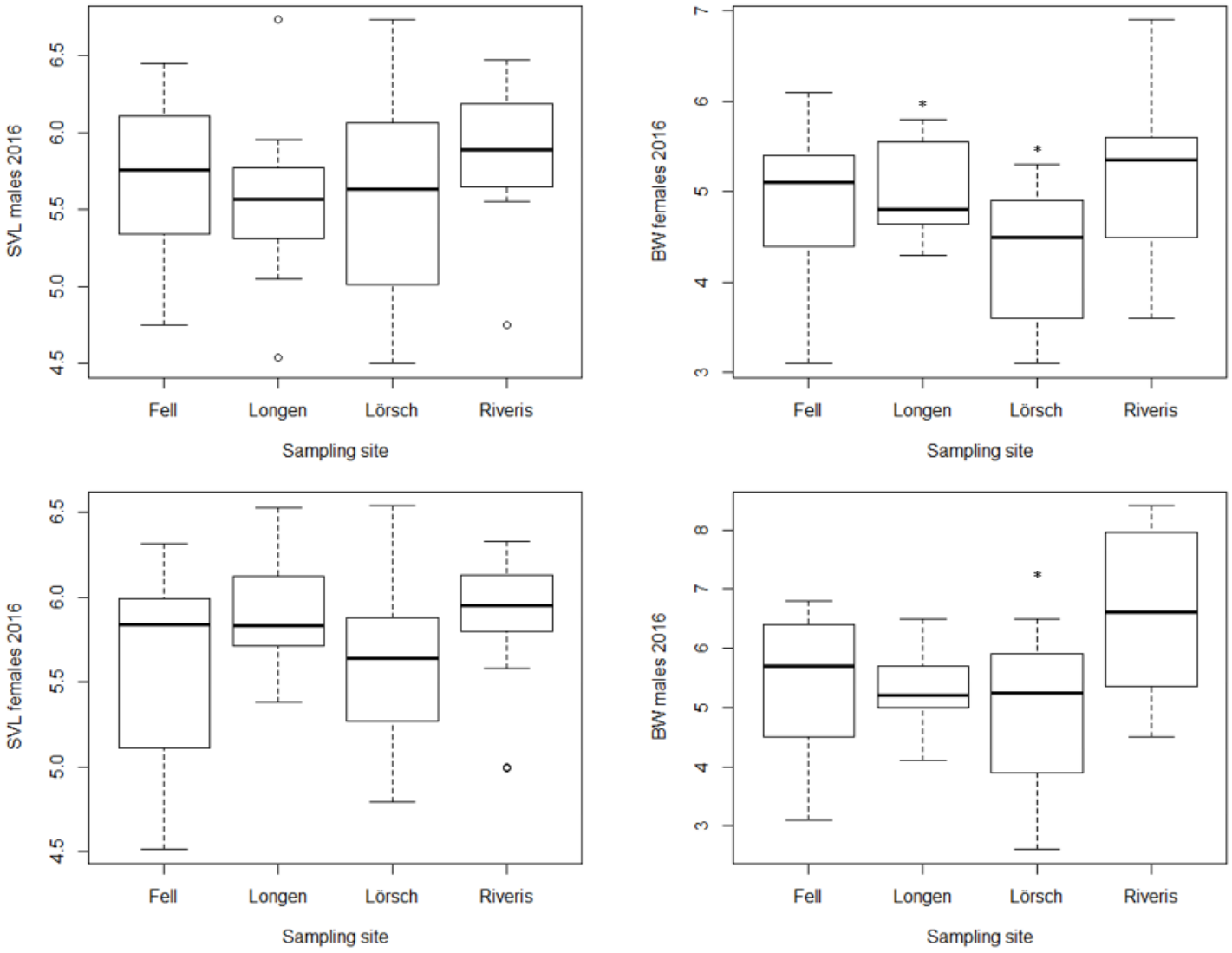


Fig. 3: Snout-to-vent-length (cm) and body weight (g) of male and female wall lizards sampled along an agricultural gradient during the year 2016. * = significant difference compared to the reference site (Riveris).

Appendix 3: Summary of relevant climate data regarding lizard activity and potential effects on pesticide leaching during the sampling period

Date	Mean Temperature (C°)	Rainfall (l/m2)	Wind (km/h)	Date	Mean Temperature (C°)	Rainfall (l/m2)	Wind (km/h)
11.04.2016	13,1	0	14	10.06.2016	15,9	0	6
12.04.2016	12,95	2,8	8	11.06.2016	15,25	13,1	7
14.04.2016	9,95	0	8	15.06.2016	14,8	12,3	11
16.04.2016	8,05	3,9	19	20.06.2016	14	3,6	14
17.04.2016	7,65	5,3	7	21.06.2016	17,35	11,3	12
18.04.2016	7	0	8	22.06.2016	20,75	3,2	6
19.04.2016	8,05	0	7	23.06.2016	24,6	0	11
20.04.2016	9,65	0	15	24.06.2016	23	0,5	8
21.04.2016	12,65	0	14	09.07.2016	20,5	0	10
22.04.2016	12,8	4,2	12	10.07.2016	22,7	0	9
23.04.2016	7,9	0,5	14	11.07.2016	20,75	0	17
24.04.2016	3,5	5,2	8	12.07.2016	18,5	0	10
31.05.2016	14,85	0,3	11	13.07.2016	15,5	17,5	9
01.06.2016	15,2	8,5	8	20.07.2016	25	0	9
02.06.2016	17,2	49,8	7	21.07.2016	23,2	41,5	10
03.06.2016	18,3	13,5	9	22.07.2016	22,9	8,9	8
04.06.2016	19	9,3	8	23.07.2016	20,2	25,6	6
05.06.2016	18,45	6,5	4	24.07.2016	21,65	2,6	4
06.06.2016	20,55	12,1	8	25.07.2016	21,4	0	7
07.06.2016	21,15	0	7	17.08.2016	20	0	9
08.06.2016	18,4	8,4	6	06.09.2016	18,8	0	6
09.06.2016	17,7	0	10	07.09.2016	20,65	0	12

Month	Mean Temperature (C°)	Rainfall (l/m2)
January	3,2	64,6
February	4,1	86
March	4,8	69,6
April	8,4	62
May	13,9	89,7
June	16,7	164,2
July	19,1	56,9
August	18,8	20,8
September	17,4	19,9
October	9,0	47,3
November	5,4	N/A
December	1,6	9,2

Saarstraße 100, 54290 Trier valentin_mingo@hotmail.com

Valentin Mingo — Curriculum Vitae

Personal information

Name:	Valentin Mingo
Date of birth:	28 April 1990
Place of Birth:	Reus, Spain

Education

02/2014 – 01/2018	**PhD Student at Trier University** • Scholarship holder of the Konrad-Adenauer-Stiftung
10/2011 - 02/2014	**Master of Science in BioGeo-Analyse at Trier University** • Thesis: "*The impact of agriculture on development of amphibians under special consideration of deformation rates"*
10/2008 - 03/2012	**Bachelor of Science in BioGeo-Analyse at Trier University** • Thesis: "*Moleculargenetic reconstruction of the dispersal history of an introduced Common wall lizard population in Passau"*
2006 - 2008	**Abitur at Institut d'educació secundària Camprils, Spain**

Working experience

01/2018 - present

Study director aquatic ecotoxicology at Eurofins Agroscience Services Ecotox GmbH, Niefern-Öschelbronn

- Planning and execution of ecotoxicological laboratory studies with aquatic organisms within the framework of admission and registration of plant protection products and chemical testing

10/2013 - 10/2016

Environmental assessments as freelancer

- **Planungsbüro ISU, Bitburg**: Detection and identification of bat fauna in relevant areas for environmental planning. Bat capture and species determination. Mapping and detection of relevant reptile and amphibian species for environmental planning.
- **Planungsbüro Neuland-Saar GbR, Nohfelden**: Detection and identification of occurring bat fauna in relevant areas for environmental planning. Bat capture and species determination.

09/2011 - 02/2013

Student assisstant at Umweltprobenbank des Bundes, Trier

- Establishment of a working marker system for the genetic differentiation of zebra and quagga mussels
- Microsatellite analyses (Gentoyping) of zebra and quagga mussels

Trier, 11 January 2018

Declaration

I declare that this thesis has been composed solely by myself and that it has not been submitted, in whole or in part, in any previous application for a degree. Except where stated otherwise by reference or acknowledgment, the work presented is entirely my own. Any thoughts from others or literal quotations are clearly marked. This thesis was not presented in the same or in a similar version to another examination board or is being published elsewhere.

Trier, 29 May 2018