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

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A B S T R A C T

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

1. Introduction

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

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

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

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

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

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

2. Materials and methods

2.1. Sample sites and study species

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

We selected *Podarcis muralis* as study species for pesticide

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

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