Deliverables 4 & 6 – Refinement of pesticide risk assessment of amphibians and reptiles based on ecology and biology of wild populations

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PEsticide RIsk AssessMent for Amphibians and Reptiles

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1) INTRODUCTION

Amphibians and reptiles are among the most threatened vertebrate taxa worldwide. About 41% (34%-51%) of amphibians and 21% (18%-33%) of reptiles are included in the IUCN categories of threatened species (Critically Endangered, Endangered or Vulnerable; IUCN, 2021; Cox et al., 2022). In Europe, nearly a quarter of amphibians and one fifth of reptiles are threatened and a further 17% of amphibians and 13% of reptiles are included among the Near Threatened species (IUCN, 2021). There are multiple reasons why amphibians and reptiles are declining, but habitat loss and degradation, followed by chemical pollution, both mainly attributable to the expansion of intensive agriculture, are commonly indicated among the major causes of herpetofauna decline worldwide as well as in the European region (Gibbons et al., 2000; Collins and Storfer, 2003; Ribeiro et al., 2009; Todd et al., 2010; Böhm et al., 2013, Arntzen et al., 2017).

Agriculture intensification that has taken place since the mid-20th century has resulted in a series of factors like habitat transformation or the use of agrochemical substances to increase crop production, which constitute a risk to wildlife populations. In particular for amphibians and reptiles, the risk is exacerbated because these animals are very vulnerable to threats at local scale due to some ecological and physiological features among which, for instance, low dispersal ability and small home ranges (Huey, 1982) and dependence on specific habitats (both terrestrial and aquatic for amphibians) and environmental requirements (i.e. thermal requirements for reptiles), varying at different life stages and throughout seasons (Bells and Russell, 2019). Furthermore, amphibians have a highly permeable skin, which increases their sensitivity to environmental alterations (Quaranta et al., 2009); many herpetological species are ground dwelling and feed on invertebrates, thus entailing higher probabilities of getting in contact with contaminants through the soil or directly from food (Vitt and Caldwell, 2014); ectothermy, and the consequent low metabolic rate, together with a simple enzyme system lead to poor chance of metabolism of absorbed chemicals (Walker and Ronis, 1989).

At present, more than 40% of the European herpetological species occur in agricultural lands (Mingo et al., 2016; IUCN 2018). Cultivated areas may be included among the habitats usually frequented by amphibians, or even in their home ranges, or they can be frequented occasionally to reach the reproductive sites or during the migrations of juveniles (e.g., Miaud and Sanuy, 2005; Fryday and Thompson, 2012; Berger et al. 2013, 2018; Lenhardt et al. 2015). Analogously, even if less information is available for these vertebrates, reptiles can occur inside fields used for basking or foraging, or during displacements, or settle there in some cases (Wisler et al., 2008; Pulsford et al., 2018; Biaggini and Corti, 2021). This occurrence of amphibians and reptiles in agricultural lands makes the effects of pesticides a potentially relevant threat for their populations (Hayes et al. 2006, Todd et al. 2010).

Despite the growing evidence pointing to the existence of pesticide risks to amphibians and reptiles (e.g. Brühl et al. 2013, Wagner et al. 2015, Mingo et al. 2016), these animals are not routinely included in the risk assessment procedures that both active ingredients and formulated products must undergo before they can be approved for marketing, which is driven by the Regulation 1107/2009 in the European Union. The publication of the EFSA Scientific Opinion (EFSA PPR Panel et al., 2018) raised concerns about the necessity of implementing protocols to evaluate pesticide risks on herpetofauna individuals; and constituted a first step towards the incorporation of these species into the regulatory risk assessment for pesticide registration. As part of the pesticide risk assessment, a characterization of the exposure of amphibians and reptiles to pesticides is necessary. In the first-tier assessment, it is common practice to estimate pesticide exposure using worst-case scenarios and simplified models, while in higher tiers the exposure assessment is refined based on a wider range of environmental parameters that affect pesticide fate and degradation and more sophisticated modelling approaches. Despite the use of pesticide models, refined exposure assessments require a good understanding of the ecological scenario in which the species inhabits, including potential exposure pathways, life cycle characteristics and biological traits that make each species vulnerable to pesticide pollution (Rico et al. 2016).

Therefore, the aim of this paper is to review those aspects of amphibian and reptilian biology, ecology and ecophysiology that should be taken into account for the high-tier exposure assessment of pesticides. By reviewing aspects of the ecology and physiology of amphibians and reptiles, we aim to improve our understanding of the presence and activities of animals in the areas that may be exposed to pesticides and, consequently, contribute to identify key exposure routes and scenarios. This review also provides recommendations for further research to complete a useful scheme for the pesticide exposure assessment of herpetofauna.

2) OVERVIEW OF AMPHIBIAN AND REPTILE LIFE HISTORY AND EXPOSURE ROUTES TO PESTICIDES

One of the main particularities of amphibians is the complexity of their life cycles. Although a wide array of different life cycle strategies exists within the group, the common mode involves the development of aquatic embryos and larvae that, after a process of metamorphosis, become terrestrial juveniles. Juvenile stage periods vary considerably among species. In European species, the juvenile stage commonly varies between one and three years, until they become sexually mature adults. Juveniles and adults are in most cases terrestrial stages, although a variable degree of dependence on water bodies exists.

Amphibian eggs and embryos develop in the aquatic environment, hence their main route of exposure to pesticides is the contact with waterborne substances. The gelatinous envelope of the egg may provide some protection against those substances that, because of their chemical properties, cannot diffuse through it (e.g. Marquis et al. 2006). Apart from exposure by contact, embryos can also become exposed to pesticides that are eventually transferred from the maternal organism. Maternal transfer of metals or persistent organic pollutants to amphibian eggs has been demonstrated (e.g. Metts et al., 2013); however, most current-use pesticides have little bioaccumulation potential, which makes maternal transfer a less relevant exposure route as compared to direct uptake from the environment.

After hatching, larvae maintain the susceptibility to exposure from contact with waterborne substances, but the risk of oral uptake comes up as soon as larvae start feeding. The relative importance of both routes is probably shifting during the larval development. In anurans, newly hatched larvae have external gills that become internalized as the animal develops. External gills significantly increase the body surface in contact with the aquatic environment and, even when gills become internal, they maintain a large exchange surface. The fact that they are covered by the larval skin probably reduces the susceptibility of dermal uptake. For example, several experimental studies have shown an increased sensitivity of newly hatched tadpoles to waterborne chemicals as compared to embryos or to late-stage tadpoles (e.g. Ortiz-Santaliestra et al. 2006, Adams & Brühl 2020). On the contrary, oral uptake of pesticides probably becomes more relevant as the development progresses and larval feeding activity turns more intense. Oral uptake may come from the ingestion of contaminated food or water (both water that circulates through the gills for breathing and what is swallowed as part of normal feeding) and from the ingestion of contaminated sediment. Larvae of many amphibian species feed on periphyton that is attached to sediments or other surfaces, and so they cannot avoid incidental sediment ingestion while feeding. Likewise, pollutants present in sediments can also be absorbed through the skin in those larvae showing benthic habits.

During metamorphosis, considered here as the period between the emergence of forelimbs to the complete tail resorption, animals do not feed because they get their energy from the reserves that are accumulated in the tail musculature. Therefore, the risk of oral uptake temporarily disappears during this stage. Once the metamorphosis finishes and most juveniles move on land, they can become exposed again through oral and dermal routes, the latter including contact with contaminated surfaces (either soils or plants) as well as overspray of those individuals standing in crop fields while pesticides are being applied (Berger et al. 2013). After metamorphosis, all amphibians are predators, feeding mostly on invertebrates that often are an important source of contamination. The diversity of exposure routes for juveniles is applicable also to adult stages. In addition, almost all adult amphibians return to the water at some point, at least for breeding, hence exposure to pesticides in the water periodically reappears. The majority of newt species also get part of their food from the water as adults (e.g. Fasola and Canova 1992), so risk for oral uptake of pesticides from the aquatic environment persists for these individuals.

Unlike amphibians, reptiles show direct development: an embryo develops inside a terrestrial egg, usually buried in the soil or hidden in crevices or hollows, from which a juvenile will hatch. In some groups like snakes, it is also common the occurrence of viviparity (e.g. vipers; Tinkle and Gibbons 1977). The duration of the juvenile stage until sexual maturity is highly variable, from less than one year in some lacertids to more than a decade in some turtles. Reptiles encompass a set of phylogenetically different groups of animals, such as turtles, squamates (including saurians, snakes or amphisbaenians), crocodiles and tuataras, with the latter two being absent in Europe. Despite the general life history pattern described above is applicable to all groups, with some exceptions like the abovementioned occurrence of viviparity, the phylogenetic variability accounts for important differences in biology, ecology and ecophysiology. For instance, some species like terrapins or water snakes have a strong dependence on the aquatic environment, spending most of their time in these types of habitats.

Reptile eggs absorb water from the surrounding environment during the entire development of the embryo (Packard et al. 1982). This means that eggs that are buried in the soils may absorb contaminants present in the pore soil water. In addition, contaminants attached to the soil particles in contact with the eggshell could also diffuse towards the egg, although this scenario is expected to be less relevant because of the lower solubility of soil-adsorbed chemicals as compared to those in the pore soil water. Unfortunately, hardly any data have been compiled about the susceptibility of reptilian eggs to absorb contaminants during their development. Only Díaz-Paniagua et al. (2002) measured organochlorine compound and heavy metal levels in chameleon egg contents from the wild, although they did not elucidate whether direct egg absorption had been the source for these chemicals. On the other hand, and as it happens with amphibians, maternal transfer of pollutants to the egg has also been proven in reptiles (Liu et al. 2019), although, as mentioned above, this is probably little relevant for current-use pesticides.

As for terrestrial amphibians, juvenile and adult reptiles may be exposed to pesticides via dermal, oral or inhalation routes. Whether the relative importance of each of these routes resembles that of amphibians is unknown, although it seems logical to expect some differences, like for instance a reduced dermal uptake motivated by a lower permeability of the skin to the diffusion of chemical agents (Weir et al. 2016). Anyway, dermal exposure because of either overspray and contact with contaminated substances may be very relevant also in reptiles, which are in close contact with the substrates and usually have small home ranges. Dietary uptake is also likely from the ingestion of contaminated prey items, while drinking water could also have its importance as a source for pollutant ingestion. However, as reviewed by EFSA PPR Panel et al. (2018), currently available data do not allow for determining how important for reptiles can be pollutant uptake via drinking water.

In a similar fashion, the lifestyle of a species can highly influence exposure patterns. Here, clear distinctions need to be made between semi aquatic and terrestrial species, but also to burrowing species. For terrestrial species, such as many snakes and lizards, exposure can take place, for example, via direct overspray (Hopkins 2006, Vyas et al. 2007, Sparling et al. 2010, Weir et al. 2010, Salice & Weir 2011). At the same time, secondary exposure through contaminated soil and plant material or even treated seeds and granules can result in chronic exposure patterns for species inhabiting treated crops (Friday & Thompson 2009, Sparling et al. 2010). In contrast, for semi-aquatic species (e.g. terrapins and some snake species), these exposure patterns are not expected. Here, leaching of pesticides into water bodies via runoff, drift or deposition of atmospheric contaminants can be expected to be of greater relevance (Sparling et al. 2010) and, as described earlier, aquatic species tend to have greater permeability towards compounds dissolved in water. However, there are also reported cases of fully terrestrial reptiles using puddles for thermoregulation or hunting (Gollmann & Gollmann 2008, Dheeraj et al. 2010). Dermal exposure as a consequence of contact with contaminated water bodies may therefore not be limited to semi-aquatic species only. Last, special consideration should also be given to burrowing species, such as those belonging to the order of amphisbaenidae. While direct overspray may be less of an issue for these taxa, their lifestyle can make them prone to chronic exposure through residues in soil.

In pesticide risk assessment conducted with terrestrial vertebrates, which routinely encompass assessment of birds and mammals only, inhalation of airborne substances is not considered as a relevant exposure route as compared with oral or dermal uptake of pesticides. This is likely to be the same case for amphibians and reptiles, although no data in this context have been published. If inhalation became an important exposure route (for instance for highly volatile compounds or because of inhalation of droplets of sprayed products), it should be considered as such also for birds and mammals. Actually, the usually higher metabolic rate of homeothermic vertebrates as compared to amphibians or reptiles would make the former more susceptible to inhalation. Consequently, the scenario of pesticides inhalation by amphibians and reptiles does not seem to provide any specific risk that should not be addressed also for birds and mammals.

3) (ECO)PHYSIOLOGICAL ASPECTS ASSOCIATED WITH EXPOSURE TO PESTICIDES

The previous section shows that amphibians and reptiles have potential to become exposed to pesticides mainly by oral and dermal routes. This approach differs from common practice in bird and mammal risk assessment, in which oral exposure is considered the predominant one, and hence assessment is mostly based on dietary uptake of pesticides. A challenging aspect in amphibian and reptile risk assessment is to implement mechanisms of exposure characterization allowing for the consideration of both oral and dermal exposures on individuals. One of these mechanisms is the integration of both routes into a single assessment using equations or models that estimate doses resulting from either route. Once this integration is achieved, exposure estimates can be refined by improving the accuracy of the different parameters included in each of the pesticide uptake equations.

A) ORAL EXPOSURE

For characterization, as part of risk assessment, of oral exposure of amphibians and reptiles to chemical substances, the US EPA developed the T-HERPS model (https://www.epa.gov/pesticide-science-and-assessing-pesticide-risks/t-herps-version-10-users-guide-risk-amphibians-and). The EFSA PPR Panel et al. (2018) suggested that this model could be used as a risk assessment tool, although some adjustments would be necessary to make it useful to some European species. They conducted a detailed assessment on how T-HERPS could be applied to different groups (see Appendix G in EFSA PPR Panel et al. 2018). Here we focus on those parameters that can be used in refinement based on information that is either available or susceptible of being generated in the future.

According to EFSA PPR Panel et al. (2018), the estimated theoretical exposure (ETE, the chemical dose resulting from oral uptake, measured in mg/kg of body weight) can be calculated as:

$ETE = FIR / bw * RUD$

where FIR is the daily food intake rate in grams, bw the animal's body weight in grams and RUD the residue unit dose in mg of chemicals/kg of food. RUD values are going to be dependent on ecological factors like diet composition or feeding habitats, which will be addressed later in this review. We will focus here on organismal factors that characterise FIR. T-HERPS proposes an allometric equation based on animals' body weight to estimate FIR of amphibians and reptiles:

$FIR = 0.013 * bw^{0.773}$

If this estimate were assumed, the only possible refinement options for the FIR would be an accurate determination of the body weight distribution within each population. However, an energy-based approach can also be used to estimate FIR, as proposed for reptiles by Fryday and Thompson (2009) based on the equation in the EFSA Guidance Document for Birds and Mammals (EFSA 2009):

$FIR = DEE / (FE * (1 - MC) * (AE / 100))$

where DEE is the daily energy expenditure in kJ/day, FE the food energy in kJ/g of dry food, MC the moisture content rate and AE the assimilation efficiency rate, with both rates ranging between 0 and 1. Fryday and Thompson (2009) collected DEE data from 67 reptilian species and ran linear regressions on the log-transformed values of DEE and body weight, obtaining linear model equations from which DEE could be predicted from the animal's body weight. The available data referred to 56 lizards (17 of which were desert species), six snakes and five chelonians (one of which was a marine turtle). EFSA PPR Panel et al. (2018), for the purposes of risk assessment of European species, recommended focusing on the equation obtained from the 39 non-desert lizard species:

$\text{Log } DEE = -0.7726 + 0.9119 * \log bw$

Further improvements of this equation can be attained if additional DEE data become available for other reptilian species.

Also, the energy-based formula to estimate FIR proposed by Fryday and Thompson (2009) is applicable to amphibians, hence developing linear models linking their DEE and body weight values would contribute to improve estimates. Unfortunately, physiological energetics studies in amphibians are not as common as in reptiles and only scattered data on amphibian DEE are available from the scientific literature. Baškiera and Gvoždík (2020) provided DEE values for adult Alpine newts, *Ichthyosaura alpestris*, within the body mass range from 2.08±0.44 to 2.45±0.59 g, as a function of the temperature as follows (data in kJ /d): 81.5±21.4 at 10°C, 116.0±30.1 at 15°C, 149.0±36.7 at 20°C and 212.0±30.2 at 25°C.

Food energy and moisture content values of the different food items was compiled from the scientific literature and summarised by EFSA (2009) (Table 1). Whereas the purpose of that compilation was to estimate food intake by birds and mammals, the values therein are applicable for amphibians and reptiles. However, a further refinement of FE and MC of specific food items (e.g. different invertebrate groups) would contribute to the accuracy of FIR estimates in herpetofauna. In this context, Rychlik and Jancewicz (2002) compiled the energetic values of some invertebrates used by shrews as a food resource. Although they referred their values to fresh weight, we have used available information on moisture content of each of those prey to estimate FE relative to dry weight and include both types of values in Table 1.

Table 1. Food energy and moisture content of different food items.

Food item	**Food energy (FE) kJ/g dry food**[a]	**Moisture content (MC) rate**[a]
Grasses and cereal shoots	17.6	0.764
Non-grass herbs	17.8	0.881
Cereal seeds	18.4	0.147
Weed seeds	21.7	0.099
Fruit	14.8	0.839
Arthropods (including caterpillars)	22.7	0.688
Fly larvae	23.5[b]	0.643[d]

Mealworm larvae	29.7[c]	0.647[e]
Soil invertebrates	19.4	0.843
Earthworms	18.1[c]	0.840[f]
Snails	12.9[c]	0.776[g]
Fish	21.0	0.737
Aquatic invertebrates	20.9	0.763
Aquatic vegetation	15.0	0.814

[a] Data from EFSA (2009) unless otherwise indicated. [b] Hawkins and Jewell (1962). [c] Ruthardt (1990). [b,c] Data retrieved from Rychlik and Jancewicz (2002) as fresh weight; for transformation into dry weight, the MC value on the right column was used. [d] Parry and Weldon (2021). [e] Vandeweyer et al. (2017), as an average of four rearing companies. [f] Conti et al. (2019). [g] Fagbuaro et al. (2006), as an average of three species.

Assimilation efficiency has been studied in reptiles, and particularly in lizards, more than in any other herpetofauna taxon. It is generally accepted that AE depends on the prey type, while the influence of other factors like meal size or body temperature is unclear. Secor and Boehm (2006) stated that larger meals would induce larger metabolic responses in part because of an increased effort needed for food assimilation; however, AE would remain constant. Regarding body temperature, the majority of studies show that it has no influence on AE (e.g. Avery 1975, Du et al. 2000, McConnachie and Alexander, 2004). Nonetheless, other studies have found an increased food assimilation efficiency in lizards as the ambient temperature raises (Buffenstein and Louw, 1982).

Among amphibians, data on AE come almost exclusively from larval stages. The available data show a much higher variability than in reptiles as a function of body temperature (Altig and McDearman 1975, Catenazzi and Kupferberg, 2018), and especially of food types (Table 2). For vegetarian anuran tadpoles, Waringer-Loschenkohl and Schagerl (2001) proposed that variation in AE among food types would be consequence of differences in the cell wall constitution among ingested species and in the uptake of inorganic materials that would help tadpoles to break down cell walls during digestion.

Table 2: Assimilation efficiency values for different amphibian (referred to larvae) and reptilian (referred to adults) species.

Group	Species / taxon	Prey / food	Temperature	AE	Reference
Lacertid lizards	Viviparous lizard *Zootoca vivipara*	Mealworms	5-20°C	0.89	Avery (1975)
	White-striped grass lizard *Takydromus wolteri*	Mealworms	26-34°C	0.844-0.888	Chen et al. (2003)
	Ordos racerunner *Eremias brenchleyi*	Mealworms	26-38°C	0.789-0.850	Xu and Ji (2006)
Non-lacertid lizards and skinks	Frill-necked lizards *Chlamydosaurus kingii*	Insects	ND	0.71	Christian et al. (1996)
	Blue-tailed skink *Eumeces elegans*	Mealworms	22-36°C	0.817-0.870	Du et al. (2000)
	Drakensberg Crag Lizard *Cordylus melanotus*	Mealworms	20-35°C	0.872	McConnachie and Alexander (2004)
Anuran tadpoles	River frog *Lithobates heckscheri*	Rabbit chow	22°C	0.538	Altig and McDearman (1975)
	Southern cricket frog *Acris gryllus*			0.702	
	Woodhouse's toad *Anaxyrus woodhousii*			0.774	
	Eastern narrowmouth toad			0.857	

	Gastrophryne carolinensis				
	Agile frog *Rana dalmatina*	*Chlamydomonas*	22°C	0.02	Waringer-Löschenkohl and Schagerl (2001)
		Spyrogyra		0.80	
	Hylidae (early larvae)	Pellets of processed grain and plant products supplemented with vitamin	24°C	~0.65-0.85	Richardson (2002)
	Hylidae (late larvae)			~0.75-0.85	
	Bufonidae (early larvae)			~0.75-0.90	
	Bufonidae (late larvae)			~0.75-0.90	
	Ranidae (early larvae)			~0.75-0.95	
	Ranidae (late larvae)			~0.65-0.80	
	Wood frog *Lithobates sylvaticus*	Benthic substrates from open canopy forests	18.6°C	0.38	Skelly and Golon (2003)
	Spring peeper *Pseudacris crucifer*			0.30	
	Foothill Yellow-legged frog *Rana boyli*	Periphyton	15.5°C	0.066	Catenazzi and Kupferberg (2018)
			19°C	0.116	
		Diatoms	15.5°C	0.101	
			19°C	0.138	
Salamander larvae	Marbled salamander *Ambystoma opacum*	Zooplankton	20°C	0.62	Regester et al. (2008)
		Chironomidae	20°C	0.69	
		Ambystoma maculatum hatchlings	20°C	0.60	

ND: no data

B) DERMAL EXPOSURE

I) AMPHIBIANS

Amphibians have a highly permeable skin, which functions as a respiratory organ and allows water and ion exchange. Because of that high permeability to the diffusion of chemical agents, dermal exposure to pesticides is acknowledged to be more relevant in amphibians than in any other group of terrestrial vertebrates. Comparative *in vitro* studies show that the pesticide percutaneous passage (expressed as cm/h) and the chemical diffusion of pesticides over the skin of the green adult frog (*Rana esculenta*) is significantly larger than that of the mammals´ skin (considering pig ear skin as model), with differences being attributed to the composition and geometry of the barrier lipids between organism groups (Quaranta et al. 2009). *In vitro* absorption tests performed with the insecticide malathion show that the percentage of absorption may range from 46 to 83%, depending on the skin model used, while differences in permeability coefficient for different compounds for the same skin model can vary by a factor of 14 (Table 3).

Table 3. *In vitro* absorption tests performed with pesticides and amphibians.

Pesticide	**Skin model**	**Exposure time**	**Absorption**	**Reference**
Malathion	American bullfrog (*Lithobates*	6h	81% (8.9x10^{-3}	Willens et al.

	catesbeianus), ventral skin		cm/h)	2006a
Malathion	American bullfrog (*Lithobates catesbeianus*), dorsal skin	6h	69% (6.5x10^{-3} cm/h)	Willens et al. 2006a
Malathion	Cane toad (*Rhinella marina*), ventral skin	6h	83% (11.3x10^{-3} cm/h)	Willens et al. 2006a
Malathion	Cane toad (*Rhinella marina*), dorsal skin	6h	77% (11.7x10^{-3} cm/h)	Willens et al. 2006a
Malathion	American bullfrog (*Lithobates catesbeianus*)[1], Harvested perfused anuran pelvic limb	6h	46%	Willens et al. 2006b
Atrazine	Edible frog (Pelophylax esculentus), ventral skin	6h	(275 cm/h)	Quaranta et al. 2009
Paraquat	Edible frog (Pelophylax esculentus), ventral skin	6h	(60 cm/h)	Quaranta et al. 2009
Glyphosate	Edible frog (Pelophylax esculentus), ventral skin	6h	(20 cm/h)	Quaranta et al. 2009

[1] This is considered a superior model as compared to cell diffusion models since it maintains the anatomic and physiologic integrity of the skin.

In vivo absorption tests are usually performed by applying the pesticide to a terrarium soil before the animals are introduced into the contaminated enclosure. Experiments performed with contaminated soils confirm that dermal exposure is a relevant exposure route for amphibians (Mendez et al. 2009; Henson-Ramsey et al. 2008), although further experiments including exposure levels relevant to field exposure scenarios and including depuration dynamics are needed to calculate pesticide kinetics.

The scarcity of pesticide exposure data for terrestrial amphibians makes it difficult to infer which of the physico-chemical properties of an active substance are more suitable to predict absorption through the amphibian skin. The octanol/water partition coefficient (Kow) of the substances is typically regarded as a determinant factor in studies on pesticide permeability and bioaccumulation, but the conclusions that are drawn about its value as a predictor of amphibian skin permeability are highly inconsistent among studies. Quaranta et al. (2009) showed that substances with a high Kow value (i.e. those showing high lipophilicity) confer a higher percutaneous passage in frog skin on a flow-through cell than those with a low Kow value, suggesting that the hydrophobicity of the substances contributes to its absorption, while the molecular mass showed no predictive value. On the other hand, Van Meter et al. (2014) found that Kow was not a strong predictor of skin permeability in amphibians placed directly on soil treated with different pesticides. These authors suggested that physiological skin reactions occurring only in living amphibians (not in *in vitro* models like that used by Quaranta et al. 2009), such as hydration, explain the differences between studies. Van Meter et al. (2015), supporting their previous study, showed that both in overspray and soil exposure treatments, the body burden and bioconcentration factors resulting from the exposure to pesticides such as atrazine, imidacloprid and pendimethalin were not related to their hydrophobicity, indicating that the role of Kow in skin permeability is not relevant in living amphibians. Differences in the relative importance of Kow to determine percutaneous passage of chemicals in amphibians occur even when comparing in vitro studies, as some studies have reported, unlike that by Quaranta et al. (2009), that flux of chemicals through the excised frog skin decreases with an increase in Kow (Kaufmann and Dohmen 2016, Llewelyn et al. 2018).

Other chemical properties related to the absorption of the substances through the soil, such as the organic carbon-water partition coefficient (Koc) or the water solubility, have been shown to be better predictors of body burdens and skin absorption than Kow (Van Meter et al. 2014, 2016). Koc indicates the capacity of a chemical to adsorb to the soil. Pesticides generally bind less to soils with a lower organic matter content, so under such conditions they become more available to be absorbed by terrestrial organisms (Wauchope et al. 2002). Van Meter et al. (2016) compared two soils with different organic matter content treated with five active substances to determine how bioconcentration in amphibians would be affected. Amphibians dermally exposed to pesticides on low organic matter soils presented higher body burdens and bioconcentration for all tested active substances, in contrast to those exposed to high organic matter soils, which presented lower body burdens and bioconcentration. Cusaac et al. (2016) found a low accumulation of pyraclostrobin in frogs exposed by contact with previously treated soils (only 5% of the predicted initial exposure), which was attributed to the quick binding of this fungicide to soils. However, preliminary findings from that study, as well as from other studies examining exposure to pesticides via soil (e.g. Henson-Ramsey et al. 2008, Van Meter et al. 2014), suggest that soil uptake may be important because the majority of observed toxicity occurred during the initial hours of exposure, when fungicide bioavailability would be still high. Regarding water solubility, polar pesticides could dissolve in the water fraction of the soil matrix becoming more bioavailable to be absorbed by amphibian skin (Wauchope et al. 2002).

Skin characteristics could be at least as important as pesticide physico-chemical properties in determining chemical diffusion, and such characteristics are highly variable across different species, and also within a single individual across different body regions. For instance, Brühl et al. (2013) found a high sensitivity of European common frog (*Rana temporaria*) juveniles to overspray with a pyroclostrobin-based formulation, which contrasts with the result of a previous assay that, with a similar methodology, had been conducted with the Great Plains toad (*Anaxyrus cognatus*) (Belden et al. 2010). They attributed this variation in sensitivity to the differences between species in skin properties, although no specific parameters were investigated in this context. According to Shah et al. (1983) differences in species sensitivity are more related to toxicokinetics-toxicodynamics, as differences in skin penetration were lower as compared to lethal doses. Likewise, absorption of contaminants, especially of most hydrophilic ones, is particularly active through the ventral pelvic region compared with the ventral thoracic or dorsal skin ones (Llewelyn et al. 2019), as the ventral pelvic region is the part of the body normally in contact with the substrate when animals are standing by, and it is there where soil water is absorbed to keep body moisture.

The collection of data about absorption of active substances covering a wide range of chemical properties is needed to elucidate the contribution of these properties to the absorption of pesticides through terrestrial amphibian skin (Table 4). Estimated burdens with living organisms exposed dorsally to pesticides, mimicking spray drift deposition, were between 23-96%, depending on the physicochemical properties of the pesticide (Shah et al. 1983).

Table 4. *In vivo* uptake and toxicokinetic tests performed with pesticides and amphibians.

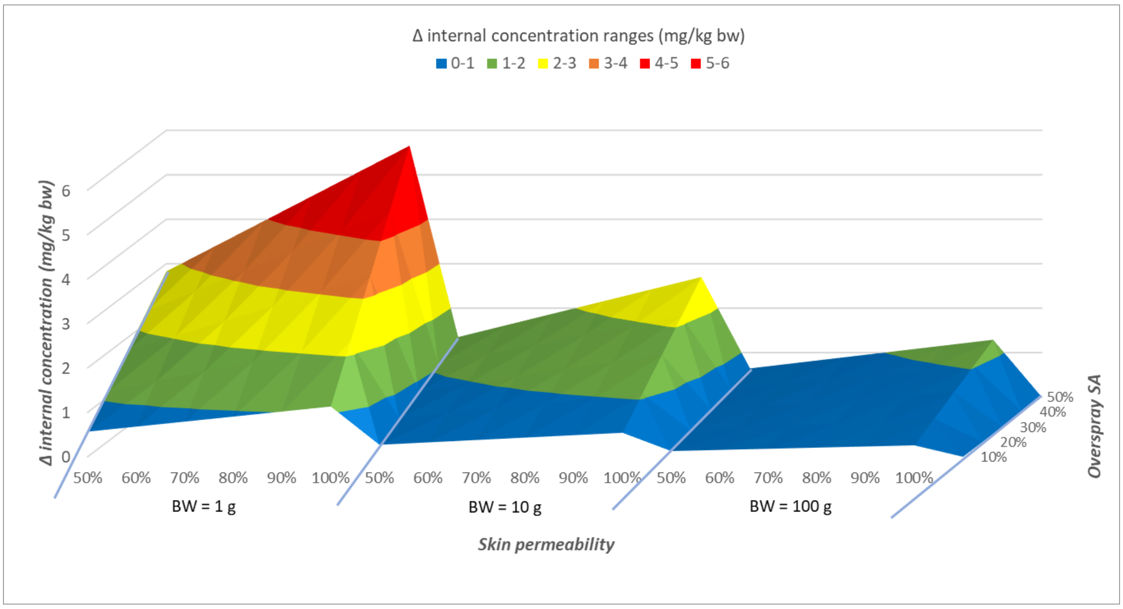
Pesticide	Species	Study type	Exposure type	Study outcome	Reference
Atrazine (radiolabeled)	American toads (*Anaxyrus americanus*)	Uptake	Soil exposure (460 µg/L)	Atrazine is uptaken by the pelvic patch and accumulates in the gall bladder and intestine	Mendez et al. (2009)
Malathion	Tiger salamanders (*Ambystoma tigrinum*)	Uptake and depuration	Soil with 50 and 100 µg/cm^2	Burdens ranged from 0.35-1.46 µg/g (brain cholinesterase activity was inhibited)	Henson-Ramsey et al. (2008)
Parathion, carbaryl, DDT, dieldrin, permethrin	Grass frog (*Lithobates pipiens*)	Uptake, body distribution	Dorsally with Hamilton syringe in a 1 cm^2 area	Burdens of 85%, 96%, 41%, 23% and 56%, respectively. 6-10% in blood	Shah et al. (1983)

				and 2-4% in liver.	
Imidacloprid, atrazine, triadimefon, fipronil, pendimethalin.	Barking treefrogs (*Hyla gratiosa*) and green treefrogs (*Hyla cinerea*)	Uptake, bioconcentration	Direct skin exposure (overspray) and indirect soil exposure. Different exposure concentrations.	Barking treefrogs: BCFs 0.1-0.9 for direct exposure, 0.03-0.8 for indirect exposure. Green treefrogs: BCFs 0.2-1.2 for direct exposure, 0.013-0.2 for indirect exposure.	Van Meter et al. 2015
Imidacloprid, atrazine, triadimefon, fipronil, pendimethalin.	Southern leopard frog (*Lithobates sphnocephala*), Fowler´s toad (*Anaxyrus fowleri*), gray treefrog (*Hyla versicolor*), Northern cricket frog (*Acris crepitans*), Eastern narrowmouth toad (*Gastrophryne carolinensis*), Baking treefrogs (*Hyla gratiosa*) and green treefrogs (*Hila cinerea*)	Uptake, bioconcentration, skin permeability factors (SPFs)	Soil exposure. Different exposure concentrations.	Wide range of BCFs (0.002-0.85) and SPFs (3.3-6.0). Soil partition coefficient and water solubility are better predictors of BCF and SPFs than Kow.	Van Meter et al. 2014
Imidacloprid, atrazine, triadimefon, fipronil, pendimethalin.	American toads (*Anaxyrus americanus*)	Uptake, bioconcentration.	Exposure from soils with different organic matter. Different exposure concentration.	Wide range of BCFs (0.1-0.61). Higher BCFs in soils with low organic matter.	Van Meter et al. 2016
Atrazine, triadimefon, fipronil	Fowler´s toad (*Anaxyrus fowleri*)	Uptake, metabolism	Soil exposure. Different exposure concentrations.	Internal concentration, clearance rates and metabolite formation. Viability of in-vitro to in-vivo extrapolations	Glinski et al. 2018

The EFSA PPR Panel et al. (2018) scientific opinion recommended the use of 100% absorption (counting half of the body area for overspray exposure) as a conservative estimate, when sufficient data is not available for the evaluated pesticide and species combination. To refine this value, further research should focus on determining the factors that determine skin permeability. Available data could serve to make some initial refinement, like the combination of substance Koc and the soil organic matter, but the currently available information does not seem to make this refinement conservative enough for a proper risk assessment.

Another important parameter in determining dermal uptake of pesticide refers to the ratio between the body surface area exposed to the pesticide, either as overspray or in contact with contaminated surfaces, and the animal's body weight. Allometric equations to estimate total body surface of different anuran and caudate models were presented, respectively, by Hutchinson et al. (1968, p.80) and Whitford and Hutchinson (1967). As for the percentage of the body surface in contact to pesticides, no specific research has been published. The EFSA opinion (EFSA PPR Panel et al. 2018) recommended a fixed value of 50%, on the basis that regardless of the animal being exposed via overspray or by contact with soil or treated plants, it will be approximately half of its body (the dorsal or the ventral half, respectively) that becomes directly exposed to pesticide. Whereas this estimation is probably too simplistic, as it approaches the body shape to a regular object, there is currently no data allowing for a more accurate data. Should information be available for both skin permeability and percent body surface area directly in contact with pesticides, that information could be directly used in dermal exposure models to refine the calculations on dermal uptake (Fig. 1).

Figure 1. Tridimensional representation of the model to estimate dermal exposure following overspray. The model is based on that presented in the Appendix I of EFSA PPR Panel et al. (2018) and estimates the increase in pesticide internal concentration because of overspray as the product between the pesticide application rate (after crop interception), the absorption efficiency through the skin and the body surface area receiving overspray (Overspray SA), divided by the animal's body weight (BW). Although the EFSA model assumes a value of 1 for absorption efficiency (100% skin permeability) and that 50% of the body surface area receives the overspray, the figure shows how the estimated increase in pesticide internal concentration would vary as a function of these two parameters for a model anuran weighting 1g, 10g or 100 g. The represented model assumes an application rate of 1 kg/ha and calculates surface area using the all-anuran equation by Hutchinson et al. (1968) i.e. $SA = 1.131 \cdot BW^{0.679}$.



The dermal uptake of pesticides is also affected by the balance between pesticide concentrations inside and outside the body. This is measured as the bioconcentration factor, or the quotient between concentration inside the organism and in the surrounding environment (e.g. concentration in soil) in steady-state conditions. Each time an animal moves over a treated field, a new balance between internal and external concentration is set, leading to increased pesticide absorption as long as movements happen throughout an evenly contaminated area. The models used in risk assessment to estimate dermal exposure consider this parameter through the transfer coefficient i.e. the body surface area in contact with contaminated surfaces per time unit. According to the model proposed by the EFSA opinion to estimate dermal uptake of pesticides from soil pore water, the transfer coefficient (α), calculated as $m \cdot d^{-1}$, would be:

$$\alpha = Cb \cdot BW / Cs \cdot 0.5 \cdot SA \cdot t \cdot 1000$$

where Cb is the concentration of the pesticide in the body (Cb), Cs the concentration in soil pore water, BW the animals' body weight, SA its body surface area and t the exposure time. The numerical factors 0.5 and 1000 are used, respectively, to assume that half of the body surface is exposed (see above) and to harmonize units among the parameters.

From this equation, the pesticide concentration in the body following dermal exposure through soil would be:

$$Cb = \alpha \cdot (Cs \cdot 0.5 \cdot SA \cdot t \cdot 1000 / BW)$$

According to this equation, for a given individual moving within an evenly contaminated soil in a given period of time (i.e. for BW, SA, Cs and t being constant), there should be a linear relationship between transfer coefficient and pesticide concentration in the body, and the determination coefficient for such relationship would be dependent on the absolute values that those 'constant' parameters in the equation (BW, SA, Cs and t) would take.

In order to properly characterize the transfer coefficient, information on the frequency of movements of amphibians within treated fields is necessary. Two variables are proposed to consider this context: time moving vs. motionless and speed of movement.

II) REPTILES

Permeability of reptile skin

Compared to amphibian skin, reptile skin is less permeable (similar to mammalian, Weir et al 2016) but not impermeable to gases, ions and water. When differentiating exposure patterns between reptiles and other terrestrial vertebrates such as birds or mammals, the first noticeable difference is the former's lack of fur and feathers. Hence, reptiles (with the exclusion of turtles, owing to their carapace) lack a protective barrier separating the skin itself from the environment, which can act as an interceptor (Chang et al. 2009). The upper layer of reptilian skin, the epidermis, is highly keratinized and acts as the first barrier (Landman et al., 1981). The level of keratinisation varies significantly among reptiles, from thin scales that are highly permeable, to very thick plates of dermal bone that are located under epidermal scales, which are termed osteoderms and are highly impermeable. However, the major barrier to skin permeability in reptiles is not keratin layer but a layer of intercellular lipids (Hopkins 2006). Species displaying reduced lipid layers are potentially more prone to uptake of polar compounds and species with thicker lipid layers are potentially more prone to uptake of lipophilic substances (Roberts & Lillywhite 1980, Tu et al. 2002, Toni & Alibardi 2007, Weir et al. 2010). As shown in *Sceloporus occidentalis* (Weir et al. 2014), lipophilic compounds can permeate through the reptile skin barrier depending also on the lipophilicity. Results of this study also showed that an initial dose of a lipophilic contaminant administered dermally remains on the outside of the skin or within the skin matrix for at least 48 hours, suggesting that a dose adsorbed/absorbed to skin may be available for uptake for several days following exposure (Weir et al., 2014).

Based on up-to date knowledge on water permeability of reptile skin, the susceptibility of water diluted pesticides across the skin of reptiles will vary at least in relation to species or population and their habitat use, environmental conditions, seasonality, life-stage, hydration status and to some extent, to ecdysis state. Skin of most of reptiles is periodically shed (Vitt and Cadwell 2014). In turtles and crocodiles, sloughing of skin is modest, but in lizards, and especially in snakes, shedding of the cornified layer results in removal of extensive sections of superficial epidermis, thus could potentially serve as a means of pollutant elimination. Also, the ecdysis cycle seems to be able to influence the sensibility of species to pesticides, presumably caused by differing skin permeability during different ecdysis phases. For example, sensibility of *Eremias argus* to pesticides varied depending on ecdysis stages, being less sensible during the proliferation phase and more sensible during the resting phase (Chang et al., 2017). Neonate reptiles do not have completely developed skin impermeability until they go through the first postnatal ecdysis. Furthermore, susceptibility to contaminants in water has been recorded to be higher in snakes with a more aquatic lifestyle, that spent more time in water compared to less aquatic snake species (e.g. Hopkins et al., 2005). It is further likely that there are regional differences in skin resistance. For example, lower water permeability rates can probably be expected on digits, tail, and head (Kattan and Lillywhite, 1989), which would differentially influence the overall permeability rate of reptile skin depending on the species' morphology.

Factors influencing dermal exposure risk in reptiles

Aside from skin anatomy and permeability, body morphology (e.g. body size and volume, relative body surface and exposed skin surface) is an important factor whilst assessing dermal exposure. Reptiles constitute a very diverse taxonomic group, characterised by high variability in body shape and size (Pincheira-Donoso et al. 2013, Watanabe et al. 2019). Within Europe, the orders of squamata (lizards and snakes) and testudines (turtles and tortoises) are represented (Glandt, 2013). For the former, many different morphologies can be found. For instance, the order can be divided into the suborders of Amphisbaenids, Snakes and Saurians. While the first two suborders share many similarities with regards to body morphology (i.e. limbless elongated bodies), Saurians are characterised by a high morphological variability, ranging from small tetrapods such as geckos, to lizards and chameleons, to limbless species such as certain skinks and slow-worms. This difference in body shapes and sizes can have strong impacts on dermal exposure. For instance, a smaller body size is generally linked to a higher skin surface area relative to body volume, creating a larger surface for absorption which can promote a comparatively higher dermal uptake of pesticides relative to the body mass (Friday & Thompson 2009, Weir et al., 2010). Smaller species can thus be expected to be generally more sensitive towards dermal uptake than larger ones. When considering the order of testudines (i.e. turtles, tortoises, and terrapins) the importance of dermal exposure changes drastically. The bony shell developed from their ribs which forms their characteristic carapace and acts as shield will probably reduce potential dermal uptake to a minimum across this surface, giving more importance to other routes of exposure (Hutchinson 1996). However, this is not true for the contact surfaces of legs, tail and head, and for further body regions in terrapins.

In a similar fashion, the lifestyle of a species can highly influence exposure patterns. Here, clear distinctions need to be made between semi aquatic and terrestrial species, but also to burrowing species. For terrestrial species, such as many snakes and lizards, exposure can take place, for example, via direct overspray (Hopkins et al. 2006, Vyas et al. 2007, Sparling et al. 2010, Weir et al. 2010, Salice & Weir 2011). At the same time, secondary exposure through contaminated soil and plant material or even treated seeds and granules can result in chronic exposure patterns for species inhabiting treated crops (Friday & Thompson 2009, Sparling et al. 2010). In contrast, for semi-aquatic species (e.g. terrapins and some snake species), these exposure patterns are not expected. Here, leaching of pesticides into water bodies via runoff, drift or deposition of atmospheric contaminants can be expected to be of greater relevance (Sparling et al. 2010) and, as described earlier, aquatic species tend to have greater permeability towards compounds dissolved in water. However, there are also reported cases of fully terrestrial reptiles using puddles for thermoregulation or hunting (Gollmann & Gollmann 2008, Dheeraj et al. 2010). Dermal exposure as a consequence of contact with contaminated water bodies may therefore not be limited to semi-aquatic species only (Table 5). Last, special consideration should also be given to burrowing species, such as those belonging to the order of amphisbaenidae. While direct overspray may be less of an issue for these taxa, their lifestyle can make them prone to chronic exposure through residues in soil.

Table 5. *In vivo* dermal/egg exposure and toxicokinetic tests performed with reptiles.

Pesticide	Species	Study	Exposure type	Study outcome	Reference

		type			
Estradiol, atrazine, endosulfan	Broad-snouted caiman (*Caiman latirostris*)	Egg exposure	50μ solution of 17 b-estradiol (1.4 ppm), atrazine (0.2 ppm) and endosulfan (0.02; 2; 20 ppm) in ethanol, one time exposure	Average 10.3% fractional weight loss in eggs and 65.7 % fractional weight in hatchlings.	Beldomenico et al. (2007)
diflubenzuron flufenoxuron	Mongolian racerunners (*Eremias argus*)	Dermal exposure	Contaminated soil (1.5 mg kg−1), exposure for 35 days	1.4–35.4 mg kg−1 of flufenoxuron and 0–1.7 mg kg−1 of diflubenzuron accumulated in the liver, brain, kidney, heart, plasma and skin. Detected alterations of triiodothyronine (T3) and thyroxine (T4) level and changes in the transcription of target genes.	Chang et al. (2018a)
Agpro glyphosate 360, Yates Roundup Weedkiller	New Zealand common Skink (*Oligosoma polychrome*)	Dermal exposure	Water solution of 144 mg/L sprayed (covered with a 4-cm layer of loose straw), exposure for 3-8 weeks	Overspray with Yates Roundup Weedkiller positively affected selected body temperatures after 3 weeks.	Carpenter et al. (2016)
glyphosate	Tegu lizard (*Salvator merianae*)	Egg exposure	50, 100, 200, 400, 800, and 1600 μg/egg, one time exposure	Significant comet assay indicating early DNA damage after 6 and 12 months of life higher at higher dosages.	Schaumburg et al. (2016)
glufosinate-ammonium; L-glufosinate-ammonium	Mongolian racerunners (*Eremias argus*)	Dermal exposure	Contaminated soil, 20 mg/kg soil weight, exposure for 60 days	Brain accumulation, detected neurotoxic effects, locomotor performance, body weight, brain weight, and brain index reduced, caused oxidative stress (after 6 and 12 months of life).	Zhang et al. (2019)

deltamethrin	spotted sand lizard (*Meroles suborbitalis*); Namaqua sand lizard (*Pedioplanis namaquensis*)	Dermal exposure	Sprayed on subjects; sprayed on soil, 17.5 and 25 g, one time exposure	Observed poisoning symptoms (every 15 min after treatment for a day, then every 4 h for 2 days), all lizards died within 2 months of treatments.	Alexander et al. (2002)
pyrethrin	green anole Lizard (*Anolis carolinensis*)	Dermal exposure	Submerged (except head) in 300 mg/L solution for 2 sec, one time exposure	Temperature influenced sensibility to pesticide.	Talent (2005)
carbaryl	black swamp snake (*Seminatrix pygaea*), diamondback water snake (*Nerodia rhombifer*)	Dermal exposure	2.5 and 5.0 mg/L solutions, exposure for 48 hours	Reduction of swimming performance with species differences.	Hopkins et al. (2005)
di-methyl phthalate, di-iso-butyl phthalate, and di-n-octyl phthalate	Western fence lizard (*Sceloporus occidentalis*)	Dermal exposure (compared to oral exposure)	Ventral skin application for 24h or 48h exposure	Residues detected in different tissues. Chemicals with lower lipophilicity had a lower dermal uptake.	Weir et al. (2014)
deltamethrin	red-eared slider turtle (*Trachemys scripta*), Chinese three-keeled pond turtle (*Chinemys reevesii*)	Egg exposure	5 µL of 0.1, 0.02, and 0.004 mg/L ethanol solution of Deltamethrin, one time exposure	Significantly decreased the swimming speed of *T. scripta* hatchlings, but not of *C. reevesii* hatchlings	Wu et al. (2016)
Beta-cyfluthrin	Eremias argus	Dermal exposure	Solution applied to skin, dose level: 0.2, 2, 20, 200 Micrograms per Gram of body weight	Thermal preference after initial temperature exposure	Wang et al. 2022

Simazine	Eremias argus	Dermal exposure	Solution applied to soil substrate, measured concentration level between 317.5 to 385 mg/kg soil, continuous exposure for 6 weeks	corticotropin-releasing factor (CRF), adrenocorticotropic hormone (ACTH)	Wang et al. 2021
3-phenoxybenzoic acid (PBA)	Eremias argus	Dermal exposure	Solution applied to soil substrate, measured concentration level of 3 and 15 µg/g soil, continuous exposure for 2 weeks	follicular epithelium heights	Chang et al. 2020
Abamectin (97%)	*Eremias argus*	Dermal exposure	Solution of 0.02 or 2 mg/kg of soil weight applied to soil substrate, continuous exposure for 30 day, pulsed application every week	average food consumption (AFC), Average maximum sprint speed (AMSS), average number of attacks (ANOA), average number of encoragement (ANOE), average processing time of food (APT), success rate of predation (SROP), Thermal preference	Nie et al. 2022

Amphibian skin is much more permeable than reptile skin, and the risk from amphibians via this pathway is therefore greater. While dermal exposure is very relevant for reptiles, dermal exposure modelling conducted for amphibians would be protective for reptiles (higher permeability and active uptake in amphibians *versus* passive uptake and lower permeability in reptiles). Thus in theory, passing an amphibian dermal risk assessment (RA) would cover the reptile RA. However, there might be substances which are many times more toxic to reptiles than to amphibians and if this is the case, reptile dermal exposure modelling could be a refinement in case of risk detected for reptiles but not for amphibians and we are providing additional relevant information here.

In an aim to compare toxicity caused by dermal exposure between reptiles and birds, Weir et al. (2010) proposed a dermal exposure model for reptiles (e.g. lizards) which could be used to estimate the internal body burden of an animal after being in contact with contaminated soil for a set amount of time. In their model, the authors proposed the following formula:

Dermal dose (mg/kg body weight) = *(kp x (0.3 x SA / H) x T x Csoil x BF) / Body weight*.

Where *Kp* is a chemical specific skin permeability coefficient (cm^2/h) and can be derived using a regression model proposed by Walker et al. (2003). Although this permeability model provided is based on mammalian skin permeability data, Weir et al. (2016) observed reptile skin permeability to pesticides to be similar to that of mammals, suggesting its suitability. This permeability coefficient could further be refined by including any new information on skin water permeability of different reptile species and under different environmental conditions. Likewise, ecdysis could be included as a worst case exposure scenario, assuming that skin permeability immediately after a skin shedding event will be higher. *SA* refers to the surface area (cm^2) of a reptile and can be derived via the use of allometric equations. In the lack of an actual lizard model, the authors proposed using a salamander model as proxy for a lizard. One third of the lizard's surface area (ventral skin) was then considered to be in contact with contaminated soil. Of course, this assumption would not cover all taxonomic groups or species. For instance, a contact area of 30% would likely be an underestimation for snakes, skinks or slowworms, while heavily overestimating exposure of tortoises, for which dermal exposure is limited to their feet. *H* represents the skin thickness of a reptile and was estimated as 1% of the body radius, simplifying the lizard as a cylinder model. The value of 1% was chosen in order to match the reported skin thickness in geckos (i.e. 0.22–0.36 mm, Bauer et al. 1989) for a given weight. Skin thickness could also be refined by known values for specific groups or species of reptiles. T is the time (h) an animal is considered to be in contact with soil. *Csoil* represents the concentration of the contaminant in soil. While Weir et al. (2016) proposed to use the application rate as proxy to derive soil concentrations, using the Predicted Environmental Concentration of the tested chemical in soil (PECsoil; mg/kg) would seem a more realistic approximation. Additionally, this approach would have the advantage that the PECsoil is used for risk assessment of soil organisms and would therefore be available for the pesticide in question (EFSA 2017). BF is a Bioavailability factor which represents the availability (uptake capacity) of the contaminant from soil. The authors proposed 3 BF, representing 10, 1 and 0.1% bioavailability. However, these BF are rather subjective and imprecise (a decision would have to be made for which one to be used). An alternative to this BF could be the use of the soil adsorption coefficient (K_{oc}; mL/g) for the tested chemical. Similar to PECsoil, K_{oc} values are standardly generated during pesticide registration and admission procedures. Studies in which the dermal uptake of contaminants (pesticides) was evaluated using amphibians demonstrated the K_{oc} to be the best predictor for uptake via the skin (Van Meter et al 2014), as opposed to e.g. the log Kow or solubility of a chemical. Since the K_{oc} is a direct measure for the adsorption of a chemical to soil particles (the lower the K_{oc}, the higher the mobility, resulting in higher bioavailability and vice-versa), it is an objective surrogate for bioavailability. Finally, the calculated body burden is normalised by the body weight (g) of the animal.

An updated uptake model based on the previously mentioned considerations could look as follows:

Dermal dose (mg/kg body weight) = *(kp x (0.3* x SA / H) x T x PECsoil /* K_{oc}*) / Body weight*

*surface area in contact with soil is suggested as 1 for burrowing species of reptiles, 0.5 for terrestrial snakes, skinks and slowworms, 0.3 for lizards and 0.01 for tortoises

Another approach is to trace the uptake of a substance in end organs and subsequently use this result to indirectly calculate the skin permeability factor (assuming the substance entered the body only via the skin surface of the animal). Chang et al. (2018a) indirectly calculated the skin permeability in the lizard *Eremias argus*, using a formula that included the lizards whole body tissue concentration of the tested substance, the soil concentration of the substance, the body weight of the lizard, the soil weight inside the tank, and the applied concentration in the soil, referring to the soil surface in contact with the lizard surface area. Results showed that flufenoxuron had e.g. a 20-fold higher propensity to penetrate through lizard skin, compared to that of diflubenzuron (Chang et al., 2018b).

Furthermore, dermal exposure may occur also in water, where aquatic snakes and terrapins would be exposed with 100% of their surface area. Similar formula, but with coefficient 1 times SA should be used, and adjusting values of Csoil to Cwater (concentration of pesticide in the water), as well as adjusting the values of bioavailability of pesticides in water.

4) ECOLOGICAL ASPECTS ASSOCIATED WITH EXPOSURE

A) HABITAT USE / SPATIAL ECOLOGY

I) AMPHIBIANS

Most amphibians spend most of their lives not in aquatic habitats (e.g. ponds and wetlands) but in the terrestrial habitat where they can be exposed to PPP. Aquatic and terrestrial habiats are both essential to sustain their normal activities and life cycles. PPP exposure can happen during seasonal migrations to and from ponds and during the parts of the year when amphibians have set up home ranges within the terrestrial habitat (Bailey and Muths 2019, Joly 2019). Amphibians can also change aquatic habitats during a single season and therefore move and stay on land around ponds (Denoël et al. 2018; Winandy et al. 2017). Finally, survival of populations is dependent on dispersal through terrestrial habitats (Cayuela et al. 2020). See also section 4.3. for discussion on timing of appliactions.

Depending on the species, terrestrial habitats can be close to ponds or at considerable distances. Species like newts do not usually go further away than few hundred metres from water bodies (e.g., Jehle & Arntzen 2000, Schabetsberger et al. 2004) while some toads can sometimes migrate to areas located several kilometres away from breeding sites (e.g., Miaud et al. 2000) but but distances can also be shorter (such as around 750m in the study of Leeb et al. (2020a), what can also depend on local configurations of habitats. Thus, in most landscapes, amphibians could be present anywhere; there are no “amphibian-free” areas.

When in the terrestrial habitat outside of the breeding season, amphibians set up home ranges within which they mostly stay. Home ranges can be located in many different habitat types and depends on habitat characteristics such as vegetation type or prey density (e.g., Indermaur et al. 2009a). Home ranges can be placed in arable land. Miaud et al. (2000) and Miaud and Sanuy (2005) used radio telemetry to study habitat use of adult natterjack toads (*Epidalea calamita*) in a semi-arid agricultural landscape in Spain. Toads were located in ditches but also in crop fields. While crop fields covered 85% of the study area, only 43% of the locations (i.e., where toads were observed during the radiotelemetry study) were within crop fields. A trend to avoidance of agricultural areas was also found in other studies on European amphibians. Salazar et al. (2016) investigated the habitat use of common toads (*Bufo bufo*) around a breeding pond in an agricultural landscape in the UK. They found toads in a distance of up to 360 m to the pond, but out of 91 detected toads, not a single individual was found directly in a cultivated field. Arable land was the most avoided type of habitat in Vos et al. (2007). During a telemetry study in a viticultural landscape in Germany, Leeb et al. (2020a) a large part of the population stayed in the agricultural landscape over large parts of the year, and only few individuals migrated to a nearby forest; this did not match the original assumption. Although toads were detected directly within vineyards, the comparison of available and used habitats showed that toads tend to avoid them as terrestrial habitat. Schweizer (2016) investigated the habitat use in an agricultural landscape in Switzerland with crop fields and pastures and found a broad use of resources (“no vegetation”, “meadow”, “crop field (vegetables)”), but preferences for embankments and the edges of fields. Importantly, toads which used flooded areas within the arable fields for breeding stayed in the fields all year round. Further radiotelemetry study could provide additional insights into spatio-temporal patterns of terrestrial habitat use by amphibians. The currently available data is limited to a few species and studies were rarely conducted in such a way that they could directly quantify PPP exposure. Unfortunately, radiotelemetry is rarely used to study habitat use by juveniles because of their small size which makes marking difficult or impossible. Therefore, there is not much information on differential habitat use by adults and juveniles. However, a methodological approach using artificial cover objects in combination with spatial capture-recapture models might be interesting (Sutherland et al. 2016). Amphibians can select habitats e.g. based on predation risk and prey availability (e.g., Indermaur et al. 2009b). There is some data which shows that suggest that amphibians can assess the suitability of water (i.e., the chemical status of it) (Smith et al. 2007), which allows avoidance of contaminated water pools, while some others indicate that they do not have the capacity to avoid contaminated soils (Mendez et al. 2009). For example, in a laboratory experiment with juvenile common toads (*Bufo bufo*), Leeb et al. (2020b) detected an avoidance behaviour against some pesticides that are frequently used in vineyards. Further research should be carried out in this direction to refine the susceptibility of amphibians to avoid contaminated patches. It should be worthwhile to quantify how often such avoidance occurs in agricultural landscapes. Furthermore, it may be worthwhile to quantify how factors such as irrigation make fields more attractive for amphibians. For example, in many areas in southern Europe, fields (e.g., corn) are irrigated and therefore the soil is moist. This may make them more attractive to amphibians than the surrounding non-agricultural land.

In general, amphibian seek a shelter during daytime while they are in the terrestrial habitat. However, this need not always be the case. Schweizer (2016) showed that during the day, natterjack toads either dug themselves into the soil or remained at the surface, implying they may be directly exposed to pesticides. The probability of being on the surface depended in a complex way on the day of season, rainfall, soil and habitat type and interactions among these explanatory variables (Schweizer, 2016). In vegetable fields, there was a probability of ~0.3 that toads would be underground during the day early in the season (day of year = 100). Later in the season (day 250), this probability increased to ~0.9. These values are averaged across soil types; toads were more likely to be underground in sandy soils than in clay soils. For example, for day 100, the probabilities were ~0.5 and ~0.4, respectively.

While telemetry studies show that some individuals spent the entire summer and fall on arable land, it is unknown which proportion of the population uses agricultural fields and which proportion uses other habitat types. The research cited above shows that arable land is often avoided. This suggests that only a small proportion of the population may be found in fields during summer. Further studies should aim to quantify the proportion of the population which uses arable fields vs. others agricultural or non-agricultural habitats. Spatial capture-recapture methods might be used to estimate amphibian density in different habitat types. Habitat-specific density estimates might be used in conjunction with the availability of different habitat types to derive estimates of the proportion of the population which uses arable land. Such estimates would be necessary for multiple species. A species such as the natterjack toad (*Epidalea calamita*) prefers open soils and is therefore more likely to use arable land than, say, the crested newt (*Triturus cristatus*) which has different habitat preferences.

Not much is known about activity patterns in the terrestrial habitat, i.e. how often are individuals moving vs. being motionless. Miaud et al. (2000) and Miaud and Sanuy (2005) described the distance moved between two subsequent localizations in radiolemetry studies. They found strong variation among individuals with some individuals moving very little whereas others moved several hundred meters. This suggests that a least some individuals move a lot. Modern radiotelemetry methods which can record the position of an individual every 15 minutes might be used to learn more about activity patterns.

II) REPTILES

Reptiles occur in multiple habitats, from rock outcrops, bushland, pastures, forests, to agriculture fields and highly modified habitats such as human settlements (Speybroeck et al., 2016). Lizards are frequently found in agricultural fields (Mingo et al., 2016 and references therein), but with different patterns of distribution and abundance (Biaggini and Corti, 2021, 2015). In general, agricultural lands are not optimal habitats for saurians and other types of reptiles (Balouch et al., 2022; Hansen et al., 2020; Ribeiro et al., 2009; Schutz and Driscoll, 2008). Further, agricultural lands may even serve as ecological traps (Rotem et al., 2013). Saurians select habitats differently in agriculture fields: generalist species select different habitats independently of the alteration while specialist species select habitats similar to their natural areas (Terán-Juárez et al., 2021). At regional and continental scales, the occurrence of saurians in European agricultural fields depends on crop extent and crop category (Bancila et al., 2023). Agroforestry and woody crops have a significant positive effect on most species (Bancila et al., 2023). Agroforestry crops include lands principally occupied by cereal crops with significant areas of natural vegetation and trees (like the typical montados and dehesas in the Iberian peninsula). The combination of agriculture and forestry enhances the persistence of reptiles (Fulgence et al., 2021; Warren-Thomas et al., 2020).

Time spent in field (PT/PD) and use of crop.

Unfortunately, no data are available about how lizards use crops in Europe. However, some data are available from other continents: dispersal movements in lizards depend on the crop matrix (Kay et al., 2016; Rotem and Ziv, 2016). Geckos can disperse when the distances to trees are short, following the sowing lines (Kay et al., 2016). Geckos behaviourally avoided farmlands, irrespective of the presence of complex habitat (Hansen et al., 2020). Lizards move from natural patches to agriculture fields before harvest, but not after harvest (Rotem et al., 2013). Movements are asymmetric between cereal and natural areas, but symmetrical between legumes and natural areas (Rotem and Ziv, 2016). Indeed, juveniles only occur in natural areas and in legume areas after harvest (Rotem and Ziv, 2016).

No data are available about how much time lizards spent in crops in Europe. The lizard *Calotes versicolor* in Pakistan avoids farmlands, but when it uses them, they are found in field margins 85% of the time (Balouch et al., 2022). To determine how much time a lizard spent in a crop, telemetry studies should be performed (Peterson and Dorcas, 1992). To our knowledge, telemetry studies in agricultural fields have not been performed for European species, but outside Europe (Balouch et al., 2022; Hansen et al., 2020; Herrera et al., 2007). Examples of telemetry studies on big lizards are (*Lacerta bilineata*: Sound and Veith, 2000; *Phrynosoma mcallii*: Wone and Beauchamp, 2003). However, telemetry studies in small lizards are impractical (Knapp and Abarca, 2009): GPS devices are to big for the animal and VHF cannot provide precise coordinates as the lizards can move while at least three angle measures are obtained to triangulate the lizard's position. The only alternatives are to use radio-tracking to localise the individual and obtain the coordinates with a GPS (Sound and Veith, 2000; Wone and Beauchamp, 2003) or to mark the animal for visual recognition and to obtain the individual's coordinates with an accurate GPS (Sillero et al., 2020, 2016).

Testudo can occur in agricultural areas and adjoining habitats. In some areas of Armenia, *T. graeca* is found in vineyards, gardens, and agricultural fields where it feeds on cultivated plants (Taskavak et al., 2004; Arakelyan & Parham, 2008); in Italy *T. hermanni* can occur close to arable fields, vineyards, pastures, and olive orchards (Biaggini & Corti, 2018). In Greece, where *T. hermanni* occur in agricultural lands too (Kati et al., 2007), Willemsem and Hailey (2001), observed some individuals eating dicotyledons recently sprayed with herbicides and basking on dead vegetation in an open area including olive groves in spring.

Analysing habitat selection and movement patterns of female Grass Snakes (*Natrix natrix helvetica*) in an agricultural landscape dominated by crops in Swiss Midlands, Wisler et al. (2008) found a clear preference for the edge habitats (embankments, dams, forest edges, and riparian zones). However, monocultures (cereals, root-crop or grass) were components of female Grass snakes' habitat during their summer activity period (with a peak in July), probably with a seasonal shift in their use: basking sites in the pre-oviposition period (25% of obs.); foraging areas after oviposition (75% of obs., after 10 July). Snakes during the study showed a unimodal daily pattern for their movements (concentrated at midday). [Mean home range was about 40 ha (range 15-120 ha). Mean distances covered per hour were 16.6 m in June, 34.8 m in July, and 3.2 m in August; movements up to 500 m were recorded to reach oviposition sites; foraging essentially along stone fences.] Analogously, in Sweden Madsen (1984) observed grass snakes (*N. natrix*) in arable lands in May-July. [info on movements and home ranges are available also in this paper]

Behaviour (e.g. burrowing or sheltering behaviour)

Lizards used to be active over the ground, moving between patches of vegetation and/or rocks (Speybroeck et al., 2016). When inactive, resting, or flying from predators, they frequently use refuges in burrows or under vegetation and rocks (Speybroeck et al., 2016).

Proportion / part of the population potentially using crop fields

No data are available indicating what proportion of lizards' populations use crops. To get such data, it is necessary to intensively survey the whole area, locating individuals inside the habitat matrix (Schutz and Driscoll, 2008). Lizards in cereal crops only occurs in the edges, while in vineyards, lizards can be found everywhere, but more abundantly in the edges (Balouch et al., 2022; Biaggini and Corti, 2021), namely when dry stone walls and vegetated field margins are present. Olive groves can be inhabited by different saurian species (i.e. *Chalcides, Lacerta, Podarcis*) when not intensively managed (i.e. low pesticide use, removal of vegetation soil cover and continuous ploughing; Carpio et al., 2017). Saurian abundance depends on the type of management: lizards are less abundant in soils without vegetation, compared to soils with grass or with natural vegetation (Carpio et al., 2017).

Time moving vs. motionless + Speed of movements

No data are available about how much time lizards spent moving inside a crop in Europe. For that, telemetry studies should necessary (Peterson and Dorcas, 1992). Balouch et al. (2022) found that hourly movement rates of lizards in Pakistan were higher in tree patches compared to grasslands and farmlands, and higher when animals moved between land cover types rather than within individual land cover types.

B) FEEDING ECOLOGY

I) AMPHIBIANS

The larval stage of caudates and anurans contrast much more than between adults. Whereas aquatic larvae of salamanders and newts forage on most small invertebrates (Braz and Joly, 1994), tadpoles of anurans are, depending on species and conditions, either open water filters or substrate grazers, consuming, phytoplankton and macrophytes as well as detritus and carcasses, including of their own species (Harrison, 1987; Loman, 2001).

In adult newts, Denoël & Demars (2008) reported a dry mass consumption of 3 mg, and 12 mg in case of oophagy. When amphibians forage on other amphibians at later life stages, particularly adults, they ingest considerably higher biomass. For instance, *Pelophylax* frogs can eat adult tree frogs of several grams of wet mass (Pille et al., 2021).

Many amphibian species can therefore ingest a variety of contaminated prey, from producers to predators, either directly when foraging in agricultural fields or outside given prey movement. As waters receive frequently pesticide run-offs, all aquatic prey can also be considered possibly contaminated in such conditions. In addition, some amphibians can forage on dead invertebrates potentially contaminated that fall on water surface and others on living terrestrial invertebrates even when they are in the water bodies.

II) REPTILES

Amphisbaenian

Due to their burrowing life-style, amphisbaenians mostly feed on soil invertebrates. Since European species, belonging to genus *Blanus*, are all small-sized, they tend to consume small prey items, mostly, insect larvae and ants (Gil et al, 1993), which are recognised by means of chemical cues (López & Martín, 1994), selected when compared to tropic availability (López et al, 1991) and manipulated with different strategies according to their size (López et al., 2013). Remarkably, sensible amounts of sediment are often ingested together with the prey (Gil et al, 1993).

Tortoises

Tortoises are almost exclusively vegetarian, consuming hundreds of plant species and fruits (Bertolero, 2015; Celse et al. 2014). While in Europe most plant species consumed are annuals, in the Sinai desert the Egyptian tortoises consume mostly perennials (Attum et al., 2021). Tortoises show selectivity in feeding and avoid consuming woody, resinous (*Pinus, Juniperus*), aromatic (*Lavandula*), or euphorbias (Bertolero, 2015). In addition to vascular plants that represent the main diet, tortoises occasionally feed on mushrooms and sometimes invertebrates (insects, snails and earthworms) (Andreu et al. 2000; Vetter, 2006). Occasional reports of coprophagy, necrophagy and geophagy indicate an opportunistic feeding pattern. Thus, tortoises consume feces from various mammal species (human, dog, jackal, rabbit, goat, or pig) which seem to be appreciated for the hair and bone fragments or moisture that they contain, as well as on carrion (Iftime and Iftime, 2012; Nikolić et al. 2016; Vetter, 2006). The occasional ingestion of soil (geophagy) is explained as a need to acquire minerals (Đorđević & Golubović, 2013; Török, 2001).

Saurians

European lizards show a wide variation in foraging habits and strategies from the mostly herbivorous (e.g. some species of Canarian *Gallotia*, Carretero, 2004) to the mostly carnivorous (e.g. genera *Anguis*, *Tarentola*) species. However, most lizard families are considered omnivorous, generalist and opportunistic foragers, eating a diversity of invertebrates, either flying or ground dwelling, along with some plant material, especially fruits, seeds and pollen, which may complete the diet during periods of low food availability (Cooper and Vitt, 2002). Intra- and interspecific lizard diet variations could be influenced by different factors such as: size (and sometimes sex) of the species, season, time constraint, and site (Carretero, 2004). Prey types are not consumed in the proportions they are available and many species tend to buffer environmental fluctuations by selecting the less abundant prey and vice-versa (Pérez-Mellado et al., 1991; Carretero, 2004). This is mediated by an ability to recognise different prey types using both visual and chemical cues. Species inhabiting arid environments or undergoing dry summers are more prone to consume prey items according to their water content.

Although lizards are often cited as exemplars of contrasting ‘‘foraging modes’’: ambush predators (sit-and-wait) and intensive foraging predators, most lacertids and skinks showed a flexibility in their foraging strategy to make use of profitable feeding opportunities; this flexibility could be particularly important if one food type becomes rare or cannot meet energetic needs (Huey and Pianka, 1981; Carretero, 2004). In contrast, European geckos mostly follow sit-and-wait strategies (Valakos & Polymeni, 1990; Hódar et al., 2006). Importantly, large Mediterranean lacertids, such *Timon* sp., are able to prey upon other vertebrates including lizards (Castilla et al., 1991) becoming placed at higher levels in trophic webs.

Diet is usually an important source of exposure to contaminants (Sparling et al., 2010), and environmental chemicals in turn may directly and indirectly affect an animal’s foraging and feeding activities, or the assimilation of foods (Amaral et al. 2012). Since European lizards occupy intermediate position in food chains, they therefore are likely to biomagnify contaminants via trophic mechanisms (Hopkins, 2000). Exposure to chemicals can (i) depress foraging by indirectly reducing prey availability in the environment, and (ii) alter the animal ability to consume and assimilate food (Walker et al., 1996). Some herbivorous species have been reported to recognise chemically prey items treated with agricultural pesticides although not necessarily to reject them.

Terrestrial snakes

Terrestrial snakes display different degrees or trophic specialization in Europe according to the species, size and trophic strategy. The largest species of lamprophids and colubrids attaining 1.5-2.5 m, such as *Malpolon monspessulanus*, *Hemorrhois hippocrepis*, *Hyerophis viridiflavus* or *Dolichophis caspius* (Díaz-Paniagua, 1976; Pleguezuelos & Moreno, 1990; Scerbak & Böhme, 1993; Luiselli, 2006) are generalist predators able to feed on a wide array of prey, including insects, reptiles, mammals and birds, which tend to be captured actively. In those species, prey spectrum enlarges and trophic level increases in the ontogeny (Valverde, 1967). Other colubrids have developed some degree of prey specialization either towards an endotherm diet in *Zamenis scalaris*. *Z. longissimus* and *Elaphe quatuorlineata* (Naullleau & Bonnet. 1995; Capizzi & Luiselli, 1997; Pleguezuelos et al., 2007), towards saurophagy in *Coronella* sp., *Macroprotodon* sp. and *Telescopus fallax* (Zuffi et al., 2010; Reading & Jofré, 2013; Speybroek et al., 2016) or towards burrowing prey in *Eyx jaculus* and *Xerotyphlops vermicularis* (Faraone et al., 2021; Speybroek et al., 2016). In contrast with most colubrids, vipers are sit-and-wait foragers of vertebrate prey which shift from lizards to small mammals and birds as main when they increase in size (Luiselli, 1996; Santos et al., 2007). Nevertheless, in dry seasons or arid regions, vipers of equivalent size will consume more lizards and less mammals (Santos et al., 2007). As an exception, *Vipera ursini* seems specialized in consuming grasshoppers (Agrimi & Luiselli, 1992).

Water snakes

In correspondence with their aquatic habits, European snakes genus *Natrix*, differ from other ophidians in consuming aquatic prey. As such, the highly aquatic *N. maura* and *N. tesellata* feed almost exclusively on anurans (frogs and toads) and fish, with some seasonal variation due to availability and juvenile snakes capturing amphibian larvae (Filippi et al., 1996; Santos & Llorente, 1998; Santos et al., 2000). The less aquatic grass snakes, *Natrix natrix* complex, are less specialized eating also anurans and fish but also small mammals and even birds (Filippi et al., 1996; Gregory & Isaac, 2004).

C) TEMPORAL PATTERNS: ACTIVITY (ANNUAL, SEASON, DAILY) AND PHENOLOGY/LIFE HISTORY

I) AMPHIBIANS

Due to the broad and varied spatial-temporal patterns of activity of amphibians they can be expected to be present in agricultural ponds and fields throughout the year. Thus, each pesticide application results in a potential exposure of amphibians. The number of exposed individuals depends strongly on the timing of the application. For example, several pesticides are applied in German agricultural fields with winter colza as early as March, so a time when many amphibians migrate to their breeding pond (Lenhardt et al., 2015). In contrast, in Central European vineyards pesticides are mainly used against fungal diseases when vine leaves are already developed. Thus, in vineyards, while there might only be a low direct exposure risk during spring migration, there could be a high risk later in the year (Leeb et al., 2020b). In general, there might be some time periods when amphibians are active in agricultural fields and no pesticides are applied. However, even then amphibians might be exposed, as European agricultural topsoil can be contaminated with pesticides even several months after the last application (Hvězdová et al., 2018). When aquatic bodies are contaminated with pesticides, amphibians might be exposed during their whole aquatic phase. Applications of pesticides on land when amphibians are in ponds can also be deterimental to them if there are risks of run-offs and then pond contamination; what may be increased during ainy periods.

Although some taxa are active during daytime, the majority of European amphibians are crepuscular or nocturnal, especially adults in their terrestrial habitat. This might reduce the risk of being sprayed directly with pesticides. However, for some insecticides an application after sunset is recommended as then the activity of some non-target species like bees is reduced. In contrast to adults, juveniles are often diurnal (SOURCE) and have therefore a comparable higher probability to be directly exposed to pesticides. Precipitation plays an important role in the daily activity in many species. For example, in

Miaud et al. (2000) the moving activity and foraging of *B. calamita* were highly dependent on rainfall in an agricultural landscape. Also Leeb et al. (2020a) found an effect of precipitation on the probability that a common toad moved.

II) REPTILES

Amphisbaenian

Adult European amphisbaenians (*Blanus* sp.) are diurnal and mainly active in spring and summer (Escarré & Vericad, 1990). Nevertheless, hibernation tends to be shorter in areas with mild climate (Valverde, 1967) while nocturnal activity has been recorded in summer. Activity, either inside the soil and on the surface under stones, depends on temperature (López et al., 1998), but is also favoured by soil moisture (Martín et al., 1990). Observations under stones tend to be bimodal with the morning peak being higher than afternoon peak (Martín et al., 1990; Gil et al., 1993). Eggs are laid underground in spring and hatch in early summer (Gil et al., 1993).

Tortoises

All three species of European tortoises are diurnal and, apart from feeding, spend a significant amount of time thermoregulating. The activity pattern of tortoises depends on the availability of plants that form their diet that varies seasonally, depending upon precipitation patterns, the availability of flowering plants and fruits, and the nutritional status of plants (Del Vecchio et al., 2011). Diet availability is also the main driver of home range size (Silveira et al., 2020). Home range varies greatly among species and individuals. In *T. graeca* it varies between 0.24-2.55 ha for females and 0.17-3 ha for males (Diaz-Paniagua and Andreu, 2015), with a maximum of 97.6 ha reported for one individual (Attum et al. 2011), while juveniles have a much lower mobility measured in tens of m^2 (Diaz-Paniagua and Andreu, 2015). Daily distances moved averaged about 50 m/day throughout the year, the maximum distances being 1019 m/day for males and 316 m/day for females (Diaz-Paniagua et al., 1995). In *T. hermanni* the average home range varies between less than 1 ha up to 5.7 ha in males and 1.4-7.4 ha in females (Bertolero 2015; Fasola et al. 2002).

Peak activities are usually in July and August in males and May and July for females (Bertolero et al., 2011; Türkozan et al. 2019). The seasonal consumption of fruits (frugivory) results in seed dispersal, which in some arid habitats is important for the maintenance of habitat diversity (Falcón et al. 2020).

Saurians

Saurians are ectothermic and rely on environmental sources for heat gain, thus their options for activity are more limited than those for endothermic tetrapods. Although most of European saurian species show a diurnal activity, there are some with crepuscular and nocturnal one (i.e., Scincidae, Gekkonidae; Vitt and Caldwell, 2014).

The spatial occurrence and temporal activity pattern of saurian species is related to temperature although optimal values and ranges often vary across species within the same community resulting in substantial phenological variation. Basking is the most observable heat-gain behaviour, even though many species (especially nocturnal ones) gain heat indirectly from surfaces they contact. Considering that sun exposure and temperatures of natural environments vary spatially and temporally, behaviours resulting in thermoregulation vary accordingly as shifting to cooler microhabitats or cooler times of day (Vitt and Caldwell, 2014). Selection and availability of appropriate microhabitats plays a fundamental role for determining the activity period (Hitchcock and McBrayer, 2006). Selection of appropriate diurnal resting sites become equally crucial not only as shelters against predators but also as heat sources in order to achieve the body temperature necessary for digestion, growth, and the subsequent activity period (Huey et al.,1989; Angilletta et al., 1999, Vasconcelos et al., 2012). Recent evidence also indicates that environmental humidity may constraint thermal activity window (e.g. Sannolo & Carretero, 2019) in those species suffering high evaporative water loss (Le Galliard et al., 2021). Moreover, climatic fluctuations are the principal force for also for cyclic dormancy: hibernation for avoidance of winter cold and aestivation for all others (Vitt and Caldwell, 2014). In continental climate, subjected to wide range of environmental temperatures during the course of the year, most saurian seek shelter where the minimum environmental temperatures will not fall below freezing and as their activity pattern includes either the hibernation or periods of inactivity determined by hypothermia (Malatesta et al., 2007).

Saurian life history

Saurian life history - as in other reptile groups - include egg (or embryo, in viviparous species as *Zootoca vivipara*), juvenile, and adult stages. Reproductive modes in saurian can be divided into two major groups: oviparity and viviparity. The former is the most common mode in the group, while viviparity occurs in approximately 20% of squamates (i.e, Scincidae, Anguidae and some species of Lacertidae family, Vitt and Caldwell, 2014). Viviparity appears as an adaptation primarily to cold climates, with short periods of appropriate conditions for activity and development of offspring (Shine, 1995), although non-thermal factors such water availability have also been involved (Bonnet et al., 2017). Mating and fertilisation in European saurian typically happens in spring, egg laying in late spring or early summer, and hatching in late summer. The climatic particularities of each location may lead to variations from this general pattern. Viviparous species inhabiting cold areas, for instance, mate right after hibernation, gestation progresses during spring, and births occur in early summer (Vitt and Caldwell, 2014). Fertilisation is internal with males having two hemipenises. Sex determination in saurian is mainly chromosomal but groups present temperature dependent sex determination (Gekkota and Scincidae, Valenzuela and Lance, 2004; Vitt and Caldwell, 2014; in Europe only demonstrated in the Phyllodactylidae *Tarentola* sp. Nettmann & Rykena, 1985; Marques et al., in revision).

Typical clutch sizes vary from 1 to 2 eggs in geckos, 3 to 20 eggs in the lacertid lizards, 1 to 18 in skinks (Vitt and Caldwell, 2014). Within each species, the number of eggs a female produce shows a trade-off with the size of offspring, which ultimately relates to juvenile survival probabilities. Annual reproductive output (calculated as the basis of clutch size, egg mass, and number of clutches per year) correlates with parental body size in an allometric way, thus the proportion of energy spent in reproduction is fairly constant across species (Meiri et al., 2012). The analysis of reproductive success (i.e., offspring number and quality) across pesticide treatments may indirectly estimate the stress experienced by adult females before and after reproduction (Gardner and Oberdorster 2016). Indeed, toxicants effects may occur later in life by lowering the energy stores that are available for reproduction through increased overall stress on the individual, decreased feeding activity, or altered metabolic pathways (Gardner and Oberdorster 2016). The ultimate effect of pesticide can be therefore measured as smaller clutch size and/or lower egg quality, or even reproduction delay or suppression (Gardner and Oberdorster 2016, Simbula et al., 2021). To our knowledge, the effects of pesticide exposure on offspring via maternal transfer of contaminants and parental stress have not been adequately investigated in reptiles yet.

In oviparous species, embryos are protected by eggshells permeable to water diffusion, which is used in yolk metabolism, indeed eggs require some sort of moisture to develop properly (Packard et al., 1982). Therefore, nesting-site selection is extremely important because of the major physiological role that environmental conditions (e.g. temperature, water or oxygen availability) play in development of eggs. The uptake of water by eggs means that soil contaminants can also be absorbed, potentially affecting embryonic development. Indeed it represent one of the main routes of chemicals exposure in the embryos of saurian along with maternal transfer during early vitellogenesis (Gardner and Oberdorster, 2016). To date, embryonic exposure routes are still poorly known and often disregarded in saurian ecotoxicology (e.g., effects of soil contaminants: Marco et al. 2004a, 2004b).

Age at sexual maturity varies from a less than one to three/four years in large lacertid lizards (Castanet, 1994; Carretero, 2006). In general, the age at sexual maturity is subjected to a trade-off between early maturation (which relates to reduced offspring size and increased chances of predation of adults) and breeding and maturing at a larger size (which results in increased pre-adult mortality and reduced number of reproductive events throughout the entire lifespan), although this can be modulated by the fact that size at sexual maturity does not necessarily correlate with age but also with juvenile growth rate (Halliday and Verrell, 1988). In addition, maximum lifespan is generally correlated with age at sexual maturity in such a way that individuals attaining the reproductive status during the first or second year of life rarely live more than five years (Vitt and Caldwell, 2014).

Snakes

Almost all European terrestrial snakes undergo winter brumation (ectotherm hibernation). This inactivity period tends to be longer than in lizards and depends on local climate and species (Pleguezuelos, 1998). Small species inhabiting mild climates and juveniles are more likely to be active in winter. Annual activity is bimodal with peaks in spring-early summer due to reproduction and early autumn (Blazquez, 1995; Pleguezuelos & Brito, 2008). Males are more active, or at least more visible, than females in spring while the opposite is observed in autumn (Pleguezuelos & Brito, 2008). Many species are diurnal and heliothermic but some may display crepuscular and even nocturnal activity by means thigmohermic thermoregulation, particularly those preying on inactive lizards, small mammals or nesting birds (Galán, 1988, Blazquez, 1995; Amat. 1998; Pollo & Puebla, 2017). Viperids also follow such strategy (Zuffi, 1999, Crnobrnja-Isailovic et al., 2007) but their annual pattern of some species is unique in included a reproductive peak in early autumn (Martínez-Freiría et al., 2010). Some colubrids, such as *Telescopus fallax* and *Macropotodon* sp., show a strong trend to nocturnality (Busack & McCoy, 1990; Speybroek et al., 2016). Finally, burrowing species are more dependent on soil temperature than on light periods (Speybroek et al., 2016).

In Europe, water snakes show similar patterns of annual activity than terrestrial snakes, with maximal activity in late spring and early autumn, but they undergo longer winter brumation (Phelps, 1978; Galán, 1988; Capula et al. 1994; Santos & Llorente, 2001). The most aquatic species are, however, less dependent in insolation having more regular daily activity and even nocturnal activity in summer (Santos & Llorente, 2001; Mebert et al., 2011).

D) SUMMARY OF ECOLOGICAL PARAMTERS AND ITS APPLICATION IN REFINEMENT

Parameter	**Taxonomic / functional group***	**Robustness / uncertainties**	**Application in refinement**	**What do we need?**
Time spent in field and use of crop.	Amphibians & Reptiles	Not too many data available	Determination of likelihood of presence within pesticide application areas	Studies quantifying patterns of differential uses of habitats, in which crop fields (where pesticides are possibly used) are contrasted with other types of habitats. Also, how crop developmental stage affects its use
Movement behaviour (direction of movements, distances per day, barriers…)	Amphibians & Reptiles	Data available for direction of movements and distances per day. Probably not so much for barrier effects	Implementation of movement of habitats on a model terrain	Studies providing data about fidelity to certain direction during migrations (from and to breeding habitats), speed of movements, resistance of different types of habitats to the passage of the animals
Hiding behaviour (e.g. burrowing or sheltering behaviour)	Amphibians & Reptiles	Data available for some species (e.g. burrowing below plant cover in the common toad)	Determination of likelihood of overspray if the behaviour happens in application areas	Relative proportion of time (and spatio-temporal parameters driving that proportion) during which animals are showing a behaviour that protects from direct overspray
Proportion / part of the population potentially using crop fields	Amphibians & Reptiles	No data available (check), but we can provide protocols to do so	Determination of likelihood of presence within pesticide application areas	Estimates of what proportion of a population can be using crop fields at different temporal scales (e.g. at a single point, along the year)

Nesting habitat (e.g. buried in (agricultural) soil, in caves, crevices, etc.)	Reptiles	Described for different species	Estimate of chances of nesting within pesticide application areas	Information about nesting sites, including proportion of nesting in cropped areas compared to others
Diet composition (food items)	Amphibians & reptiles	Data for different species available in literature (a VMG was conducted on that)	Not directly applicable to pop models, but can be used to quantify effects following oral exposure	Proportion of different food items in diet
Food regime / foraging frequency	Amphibians & reptiles (especially those with less regular regimes, e.g. snakes)	Probably not too much data (some data on food intake rate compiled in the VMG referred above)	Not directly applicable to pop models, but can be used to quantify effects following oral exposure	Frequency of feeding, food intake rates per day and at different temporal scales, maximum amount of food per feeding bout
Time spent feeding on crop fields / Proportion of the diet obtained from crop fields	Amphibians & reptiles	Probably not too much data	The variable applicable to pop models is habitat use (see above), but this can used to refine it, and anyway to quantify effects following oral exposure	Proportion of foraging time that animals spend on crop fields or proportion of their diet that they obtain while in crop fields. If possible differential diet while in crop fields or off-crops. Consider differences because of crop developmental stages
Daily activity patterns	Amphibians & Reptiles	Described for many species (e.g. no direct exposure/overspray for	Determination of likelihood of being present in crop field during application times	Hours of activity or movement, especially on land. Consider differences because of season or environmental parameters

		nocturnal species in case of morning applications)		
Annual activity patterns	Amphibians & Reptiles	Occurrence and activity throughout the year described for different species and life stages	Determination of likelihood of being present in crop field during application times	Seasonal activity patterns, including determinants for the beginning and end of breeding periods, migrations, etc. Consider factors influencing, like rainfall periods, etc.

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