Deliverable 2 – Needs to develop ecotoxicity methods for reptiles

CA18221 – PERIAMAR
PEsticide RIsk AssessMent for Amphibians and Reptiles

Authors: Giulia Simbula[1], Miguel A. Carrertero[1,2], David Du Pasquier[3]

[1] CIBIO - Centro de Investigação em Biodiversidade e Recursos Genéticos. Universidade do Porto Campus de Vairão. Rua Padre Armando Quintas, nº 7. 4485-661 Vairão, Portugal
[2] Departamento de Biologia, Faculdade de Ciências, Universidade do Porto, 4099-002 Porto, Portugal
[3] Laboratoire Watchfrog. 1 rue Pierre Fontaine, 91000 Evry, France.

Funded by the European Union

Summary

This document constitutes the deliverable # 2 of PERIAMAR. Whereas the initial plan was to focus the need for developing test methods on both amphibians and reptiles, the activities conducted as part of COST Action PERIAMAR have allowed to develop a risk assessment scheme for amphibians where the need for test methods is not identified (see deliverable #13 of the Action). Consequently, the focus on needs for test methods is placed on reptiles, for which the availability of information is much lower than for amphibians and for which the need of generating more toxicity data is pressing. This deliverable summarizes the situation of reptiles relative to the risk assessment and lists the needs that, after activities and discussion maintained during the PERIAMAR COST Action, are identified.

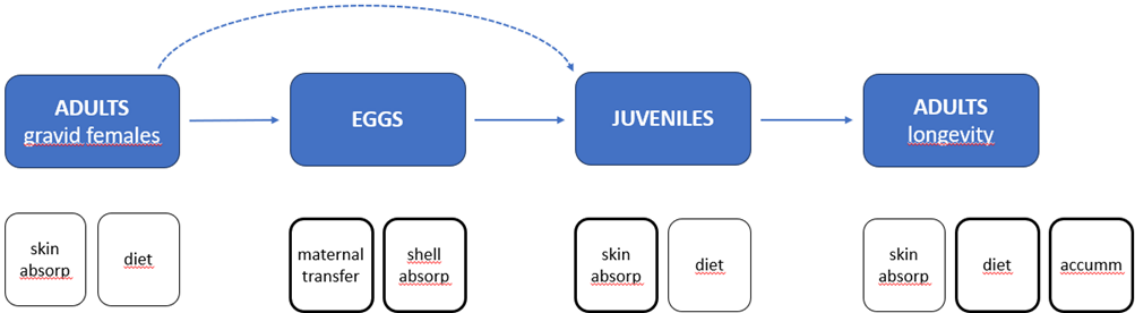
The situation of reptiles relative to risk assessment

The most relevant issues for the under-representation of reptiles ecotoxicological studies and the paucity of toxicity data are the absence of test guidelines and the lack of appropriate model species. Reptiles present a high diversity ecological requirement (Hopkins, 2000), they can inhabit terrestrial, freshwater and marine environments. Moreover, their life history greatly differs from that of other vertebrates: compared to amphibians for example, they lack a larval stage, but internal fecundation (and for some species viviparity) makes females to remain gravid during long periods. Furthermore, many species are incompatible with laboratory studies because of their size, life span or rates of maturity.

Reptiles can be exposed to contaminants through various routes, based on their life stage (Fig. 1). Adult, for example, can be exposed by: (a) inhalation (Doya et al., 2020), (b) ingestion of contaminated food or soil (Mingo et al., 2017; Rosati et al., 2023) and (c) absorption from the skin (Weir et al., 2016; Mestre et al., 2019). However, since reptile species may occupy different trophic levels and exhibit different feeding ecologies, the intensity of oral uptake could be difficult to generalize. Biomagnification might pose a high risk for high trophic level reptiles (predators such snakes and large lizards) (Sparling et al. 2010); scavenger reptiles (such freshwater turtles) may experience increased levels of exposure due to ingestion of levels of contaminants that were lethal to prey (Hopkins, 2006). In a similar way, herbivorous reptiles (such land tortoises) are sensitive not only to contaminants found in plants, but also to pesticides accumulated on their surfaces. In addition, regarding the skin absorption, it must be taken in account the existence of aquatic species with amphibious life styles (e.g., water snakes and freshwater turtles). These species are expected to be exposed to pesticides through skin not only in their terrestrial habitat but also in the aquatic one (Dos Santos et al., 2021). Until now, the dermal and oral routes of exposure were considered the most significant in playing a role in reptile ecotoxicology (Hopkins et al. 2006, Sparling et al. 2010, Weir et al. 2010). However, in the last decade, field and experimental studies have demonstrated that the effects of exposure to chemicals can be quite severe also during pregnancy and early life stages, when reptiles seem to be most vulnerable. Gravid females will indeed buffer the influence of varying environmental parameters on embryos. This means that mothers will prioritise offspring's needs for thermoregulation and hydro-regulation even against their own needs (Beltrán et al., 2021). As such, gravid females are more sensitive to dehydration and choose different thermal environments and microhabitats, increasing their exposure to pesticides. Bulky bellies, which make the skin more permeable, are likely to increase exposure (Lourdais et al., 2015). Additionally, in viviparous species, gestation is long (Díaz-Paniagua et al., 2007), which could increase exposure time of the embryo to any kind of contaminats. In this case,

maternal transfer may be the major route of exposure during development. However, in oviparous species maternal transfer represents a minor contamination route compared to the uptake of environmental chemicals from the surrounding soil or matrix of nest materials (Sparling et al., 2010). Unlike avian eggs which have a thick calcareous outer layer, the soft porous eggs of the lizards (and likely snakes and turtles) constantly exchange water and gasses with the surrounding environment (Marco et al., 2004; 2005). During this process, contaminants are easily transferred from the external environment into the eggs and strongly interfere with the physiological dynamics of embryo development, including regulation of gene expression (Odetti et al., 2023). Finally, after hatching, small juveniles are exposed to the same routes as the adults, but because they are more sensitive to dehydration (higher surface to volume ratio) (Sannolo and Carretero, 2019), they select different microhabitats. In addition, the low mobility of juveniles makes them more vulnerable than adults to agricultural treatments (Civantos, 2000; Civantos and Forsman, 2000). Moreover, the high growth rates of these animals result in a lot of prey items consumed, which also might increase the exposure to pesticides.

Fig. 1: Scheme of the life stages of reptiles and possible route of exposure to pesticides



There are diverse ways to determine stress level in reptiles at any life stage: changes in body condition, behaviour, tissular and enzymatic alterations, reproductive success, presence of abnormalities and survival. Different ecotoxicological studies on reptiles have adopted some of these endpoints (e.g., LC10, LC50, LD50, antioxidant system and genotoxicity, reproduction) through concentration and dose level lab experiments to determine pesticide effects. Although some of the outcomes of these studies have enabled to narrow the gaps on knowledge of the effects of several pesticides on reptiles, the majority lack methodological consistency and essential information such as temperature and hydroregulation. In this vertebrate group, thermal and hydric environment play a significant role: adults are thermoregulators (and hydroregulators), while eggs are thermoconformers absorbing water when recently laid. Thus, temperature and moisture can be critical parameters in determining the effects of pesticides (Weir et al., 2015).

In order to build a risk assessment for reptiles, more toxicity data for reptiles are fundamental to cover and prioritize several ecotoxicological aspects: i) it would clarify if birds are good surrogates for reptiles, thus precluding the need to perform reptile toxicity tests for each individual pesticide. In some cases, reptiles may be more sensitive than birds (e.g., pyrethroid insecticides; Weir et al., 2010). Conversely, in some instances, birds could be more sensitive than reptiles, thus, using birds in an ecological risk assessment would overestimate risk to reptiles (Weir et al., 2015). Remarkably, since birds are phylogenetically nested within reptiles (closely related to crocodiles), diverse ecotoxicological responses can be observed across different reptile groups. Increasing the available toxicological data for both groups, but mainly reptiles, would allow to better understand the relationship between reptile and avian toxicity for chemical classes or modes of action and whether reliable relationships could be

generated. ii) Implement exiting and exhaustive population models with toxicity data to extrapolate population persistence of specific protection goals (SPGs) for reptiles. iii) Increase ecotoxicological studies (exposure-based) on some of the unique characteristics of reptiles compared to other vertebrates, such as: chromatographic studies related to toxicity data in long-lived species, or uptake of contamination by the egg/embryo through the soil. Controlled laboratory exposure and the use of appropriate physiological biomarkers will be essential for early detection of xenobiotics effect (e.g. on growth, development, reproduction or survival).

Steps for developing a toxicity test method with reptiles

Considering aspects ii and iii identified in the last paragraph of the previous section, the necessity of test methods for toxicity assessment in reptiles could become a major need in order to generate new data. This opposes to the EU regulation in terms of animal welfare (e.g. Directive 63/2010 stating that the use of vertebrate testing in scientific assessment should be kept to a minimum); however, there may be a need to developing these test methods at least to be used for some time to increase the body of knowledge of reptile ecotoxicology. Once the existing dataset is sufficiently comprehensive, robust extrapolation methods can be developed, enabling the cessation of live reptile use in toxicity testing.

The following steps are suggested when developing test methods for reptiles:

Step 1: Election of a test method amendable for standardization, for which the following must be taken into account:

- Data from standardized methods have a much better scientific and regulatory acceptance.
- Standardized methods are needed for useful comparisons of the toxic effect between chemicals.
- OECD test guideline guarantee the mutual acceptance of data (across countries/regulators), lowering the number of animals used. “Tested once, accepted everywhere”.

To achieve this, the test method outputs must be transferable, and the test itself must have the ability to provide reproducible and relevant data.

Step 2: Election of a test species, with the following considerations:

- Commercially available (if any).
- Existing methods for long-term culturing in the lab. In this context, there are some clearly advantageous models like *Sceloporus occidentalis*, *Eremias argus* or *Podarcis siculus*, both of which have been used before in reptilian toxicity testing.
- Choose an adequate model species to represent the reptilian phylogenetic tree. Reptilian taxonomy is complex, as reptiles are actually not a monophyletic group. However, some clades are clearly non-relevant (e.g. crocodilians) or less relevant than others in terms of the risks that pesticides pose to reptilian EU fauna.

Step 3: Define the “toxicity” to address. This must be driven by the scientific and regulatory need, or in other words by the protection goal. As set in the EFSA Scientific Opinion (EFSA PPR Panel et al., 2018), specific protection goals for reptiles are individual survival and population persistence. That means that acute test reporting mortality-related endpoints, or long-term tests reporting endpoints relevant to population dynamics (e.g. reproduction) should be prioritized.

Step 4: Define the endpoints. This is highly related to the previous. The following endpoints have been identified from chronic toxicity studies (OECD, 2018), many of which can be retrieved from existing reptilian toxicological literature or are suitable to be recoded:

- Body weight and body weight change.
- Food/water consumption.
- Sensory activity, grip strength and motor activity.
- Haematological responses.
- Clinical biochemistry tests.
- Necropsy findings including organ weight data.
- Diverse histopathological findings.

The endpoint selection should of course be driven by the type of test and the protection goal. In this context, the battery of options seems wide enough to find an appropriate tool. The main challenge lies, however, on the definition of the previous steps.

Step 5: Establishing a Standard Operating Procedure (SOP), which should consider:

- Number and sex of the animals
- Developmental window of exposure / Duration
- Route of administration (Oral, Dermal, Inhalation…)
- Dose (maximum tested concentration, number of dose, spacing factor)
- Housing and feeding conditions
- Selection /preparation of the animals
- Dailly observations
- Quantification of the endpoints (measurement, necropsy, histopathology…)
- Data analysis and statistical approach
- Result interpretation

Step 6: Optimize the SOP and fix all parameters (OECD, 2005), through:

- Testing of reference chemicals representative of the types of substances for which the test method will be used.
- Each element of a test (for example chemical exposure time, histopathology, clinical chemistry, etc.,) needs to be carefully explored and evaluated to determine the optimum conditions/details.

Step 7: Validation of the method, for which the Principles And Criteria For Test Method Validation (OECD, 2005) should be followed:

- The rationale for the test method should be available.
- The relationship between the endpoint(s) and the phenomenon of interest should be described.
- A detailed protocol should be available.
- The intra-, and inter-laboratory reproducibility should be demonstrated*.
- Test method's performance based on the testing of reference chemicals*.
- The performance evaluated in relation to relevant information from the species of concern, and existing relevant toxicity data.
- All data available for expert review.

The two criteria marked with an asterisk (*) are addressed by means of multilaboratory ring tests.

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