CHAPTER 12

Genotoxic, Biochemical and Physiological Biomarkers Triggered by Agrochemicals in Neotropical Anuran Species

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12.1 The Use of Agrochemicals in the Neotropical Region

Since the "Green Revolution" started 50 years ago, modern agriculture has been associated with a large use of agrochemicals, such as herbicides, insecticides and fungicides, among other agents.[1,2] In the group of the most employed agrochemicals applied in the Neotropical region, we can mention four classes of herbicides: (1) non-selective post-emergence [*e.g.*, glyphosate (GLY) and paraquat], (2) selective pre- and post-emergence [*e.g.*, atrazine (ATZ) and clomazone (CMZ)], (3) selective post-emergence [*e.g.*, 2,4-D, dicamba (DIC), glufosinate-ammonium (GLA) and picloram (PCM)] and (4) synthetic compounds termed as imidazolinone herbicides, including selective pre-emergence groups [*e.g.*, flurochloridone (FLC)], to name a few. Besides, other agrochemicals such as organochlorine (OCs), organophosphate (OPEs), pyrethroid (PYRs) and neonicotinoid (NEOs) insecticides should also be mentioned in the list.[3,4]

In Latin America, these agricultural practices have neither been subject to a critical evaluation nor strict official regulation procedures. Also lacking is adequate information regarding their impact and mitigation measures to be implemented in countries where agrochemicals are intensively used.[5] These deficiencies, coupled with deforestation and fragmentation of natural habitats produced by the current agricultural model, lead us to think about the possible risk to the health of the human population, environment and other non-target living species, such as anurans.[5]

It is well known that amphibians are the most vulnerable group of vertebrates, with approximately 41% of the species threatened worldwide.[6,7] In this context, the contamination of ecosystems (both terrestrial and aquatic) by agrochemicals stands out as a major stress factor influencing the global decline in amphibian populations.[7–9] Several studies have reported the adverse effects caused by these agents in anuran populations both globally and in Neotropical species in particular. The range goes from disruption in trophic relationships and alterations in survival rates to metamorphosis in those populations with genetic disorders.[8,9] In this regard, amphibians are excellent bioindicators of environmental health status[7,8] and are considered "new-age biological models" for ecotoxicological assessments of the health of a given ecosystem.[10–12]

The use of biomarkers in native non-conventional anuran models allows for obtaining a toxicological profile of the biota's response to environmental contaminants. Bearing in mind that different aquatic species are not equally susceptible to the same pollutant and even the same species is not equally susceptible throughout its life cycle, underpins the importance of the use of different biological models.[13,14] This, in turn, highlights the interactions between biotic matrices and environmental stressors that will enable us to reveal realistic scenarios in risk assessment programmes.[3,4] When evaluating the adverse effects of agrochemicals in anurans, it is useful to apply different biomarkers such as molecular, biochemical, histological and physiological ones, which can provide more accurate information during the complete life cycle of this taxon. This approach will help us better understand the effects of these environmental stressors on the decline of amphibian populations.[8,13,14] If we consider the concept of biomarkers on a scale of ecological levels (see Figure 12.1), we can find three large groups of biomarkers that include physiological, cytogenetic and biochemical levels (see Sections 12.2.1, 12.2.2 and 12.2.3). In turn, these groups of biomarkers can be integrated for their application in the evaluation of adverse effects in reproductive scales (see Section 12.2.4).[15]

Neotropics are the most biodiverse regions on our planet and have the largest number of anurans worldwide, with approximately more than 3030 species reported so far.[16] As mentioned previously, this is coupled with the fact that the Neotropical region is the largest zoogeographical area impacted by modern agriculture associated with the use of genetically modified organisms and agrochemicals at a large scale.[1] In this context and focusing on the importance of evaluating biomarkers in native anurans, studies on Neotropical species have only covered 1.2% of the total 36 species evaluated so far. We have noticed a growing trend in the use of biomarkers in Neotropical anurans in the last 10 years (see Figure 12.2). These studies consider different life stages of anurans (embryos, tadpoles and adults), demonstrating the effectiveness and the importance of the use of different biomarkers for evaluating pollutant(s) exposure, detecting adverse effects at relevant environmental concentrations and for environmental risk assessment programmes.

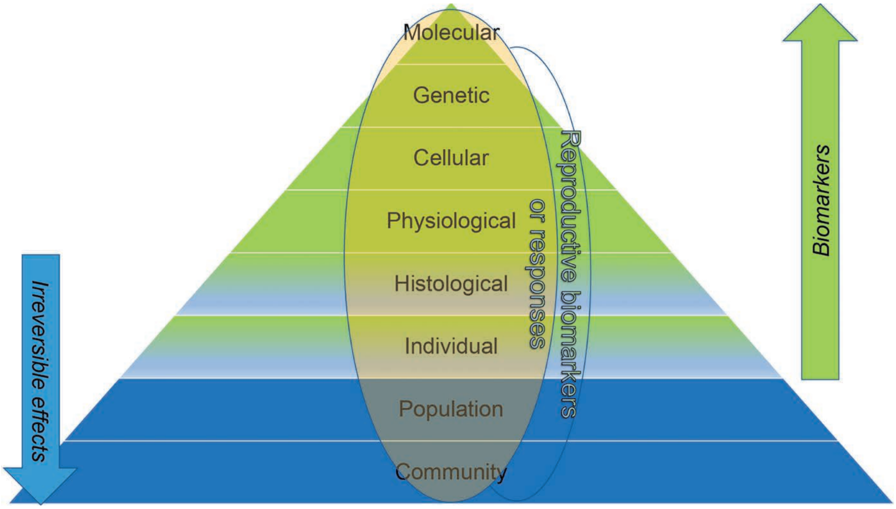

Figure 12.1 Different levels of biological organisation for biomarkers evaluation: a multibiomarker approach concept. The reproduction biomarkers or responses cover all levels.

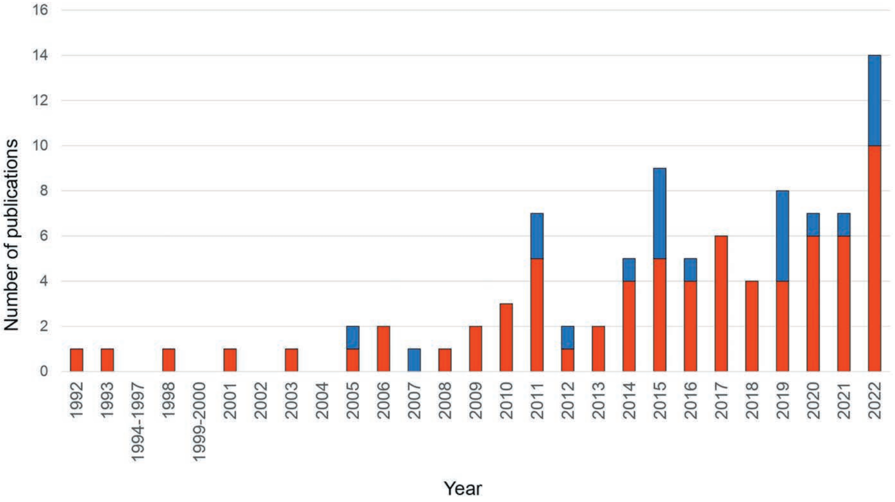

Figure 12.2 Trends in the use of biomarkers during the period 1992–2022 in Neotropical anurans according to scientific databases. Cobalt blue section bars represent biomonitoring studies, while red section bars represent laboratory bioassays.

Biomarkers of exposure, effect and susceptibility are needed to relate the presence of pollutants in the environment with their action in organisms.[3,4,13] In this context, they can assist in assessing the health status of amphibian populations by acting as sublethal endpoints of intoxication.[13] The different biomarkers used in Neotropical anurans in the next sections of this chapter will be listed according to the type of agrochemicals involved. In addition, some important concepts about the advantages and disadvantages of using biomarkers at different ecotoxicological scales will also be mentioned and discussed.

12.2 Biomarkers in Neotropical Anurans

12.2.1 Physiological Biomarkers

Although recently the use of amphibians as bioindicators or sentinel species has grown in the field of environmental monitoring,[17,18] studies of physiological biomarkers aimed at evaluating the ecotoxicity of contaminants are still scarce.[19–28] Physiological biomarkers can involve cardiac alterations related to crucial mechanisms such as homeostasis, metamorphosis, growth and metabolism. Specifically, cardiac effects involve relaxation of the heart, heart rate alterations, reduced atrium size and organ malformation, as observed in two well-known anuran experimental models such as the American bullfrog *Aquarana catesbeiana* tadpoles and the African clawed frog *Xenopus laevis*.[21–23,27,28,31,32] Another group of physiological biomarkers are endocrine-disrupting chemicals (EDCs), which are actively present in several pesticides and affect reproduction and development.[29] It is worth mentioning that amphibians' development is highly susceptible to environmental contaminants since thyroid hormones and their receptors are frequently altered by many toxicants.[29] The studies with amphibian model species showed that distinct agrochemicals have decreased metamorphic rates.[29,30] Table 12.1 summarises the main physiological biomarkers of amphibians applied in toxicological studies of agrochemicals.

12.2.1.1 Case Study: Herbicides

Studies on herbicides showed that cardiac physiological biomarkers reflect the effects of agrochemical use. Specifically, some pesticides have been reported as cardiotoxic in model amphibians.[22,23] Focusing on Neotropical species, EDC biomarkers have been shown to be effective after exposure to commercial formulations of butachlor (Machete EC) in the Neotropical cane toad *Rhinella marina*. This report evidenced alterations in the development of the thyroid gland and consequently decreased the rates of metamorphosis at butachlor concentrations ranging between 0.002 and 0.2 mg L^{-1}.[26] Another study employing the snouted tree frog *Scinax nasicus* and the two-coloured oval frog *Elaschistocleis bicolor* reported that the herbicide DIC in its formulation Cowboy Elite Surcos® (20% active ingredient) increased the production of thyroid hormones in concentrations ranging between 0.01875 and 20 mg L^{-1}.[25] These studies demonstrate the high potential of endocrine biomarkers to be used as sentinel indicators in response to agrochemicals.

12.2.1.2 Case Study: Insecticides

Cardiac effects have been detected in Neotropical species, but to the best of our knowledge, only one study has been carried out.[24] This study shows a decrease in cardiac activity (bradycardia) in the common lesser "escuercito", also called the American ground frog, *Odontophrynus asper* (= *O. americanus*) tadpoles exposed at sublethal concentrations (between 0.1 and 10 mg L^{-1}) to the commercial insecticide pyriproxyfen Dragon® (2% active ingredient of the pyridine-based pesticide).[24]

The above-described review demonstrates that although physiological biomarkers are, in fact, very sensitive, early and effective indicators of environmental changes, their use in ecotoxicological studies is still quite limited. It is worth noting that some authors suggest evaluating, for agrochemical exposure, novel biomarkers in adults, related to permeable and highly vascularised skin, which acts both as an osmoregulatory and as a respiratory organ.[32] However, the wide variation among the different effects of agrochemicals on the anuran models demonstrates their sensitivity but lack of specificity as biomarkers to stressors. This limitation is even

Table 12.1 Physiological biomarkers commonly used for the evaluation of agrochemicals in anurans. Letters indicate: commercial formulation (F), pure active ingredient (AI), herbicide (H), insecticide (I), embryos (E), tadpoles (T) and adults (A).

Agrochemical							
Type	**Presentation**	**Class**	**Exposure stage**	**Exposure period (days)**	**Species**	**Biomarker**	**Ref.**
Glyphosate	F	H	T	2	*Aquarana catesbeiana*	Cardiac: heart rate, ventricular inotropism and chronotropism	22
Chlorpyrifos	AI	I	T	5	*Xenopus laevis*	Cardiac: heart rate	31
Methyl parathion	F	I	T	2	*Aquarana catesbeiana*	Cardiac: heart rate, ventricular inotropism, ventricular mass	23
Azocyclotin	AI	I	T	21	*Xenopus laevis*	Endocrine: metamorphic rate	30
Pyriproxyfen	F	I	T	2	*Odontophrynus laevis*	Cardiac: heart rate	24
Dicamba	AI	H	T	2	*Scinax nasicus*, *Elachistoclei bicolor*	Endocrine: thyroid hormone levels	25
Imazapyr	F	H	E	4	*Xenopus laevis*	Cardiac: oedema	28
Butachlor	AI	H	T	12	*Rhinella marina*	Endocrine: metamorphic rate, thyroid development	26
Chlorpyrifos	AI	I	A	45	*Xenopus laevis*	Osmoregulatory: decreased function	32
Glyphosate	F	H	E	3.5	*Xenopus laevis*	Cardiac: heart malformations	27

more significant when it comes to Neotropical species. In addition, the present lack of knowledge limits our understanding of the toxicodynamical mechanisms involved in agrochemicals.[25,26]

12.2.2 Cytogenetic Biomarkers

The term *genotoxicity* refers to any physical or chemical agent capable of inducing damage in the chromosomes or DNA, altering its normal structure. Moreover, the term *cytotoxicity* encompasses the toxic effects that cause damage and cell death in a target tissue or organ.[3,33] Different bioassays can be performed to visualise and quantify these alterations and have an important role to play in the prediction potential of certain cytotoxic xenobiotics and genotoxicity, which consequently may trigger a carcinogenesis process. Cytogenetic bioassays have the advantage of being reproducible and, depending on the type of tissue, pointing out the possibility of reparation of certain induced damages.[34] It is often necessary to employ a set of bioassays to cover different biological systems, as there is no single assay that detects all genotoxic agents or all types of cyto- or genotoxic damage.[35] In anurans, the first cytogenetic studies were performed 30 years ago using *micronucleus* (MN) and *single-cell gel electrophoresis* (SCGE, comet assay) assays.[36,37] Specifically, for Neotropical anurans, the first MN studies date from 1993[38] but only from 2005 onwards were they massively employed. Most used techniques in Neotropical anurans include MN assay, SCGE assay, mitotic index and cellular viability. New studies have been encouraged to propose novel biomarkers applied to cyto- and genotoxicity studies in Neotropical anurans, such as the viability technique[39] and heterophil/lymphocyte (H/L) ratio.[40]

12.2.2.1 Micronucleus Assay

This methodology allows for the evaluation of the damage induced by a xenobiotic at the chromosomal level and can be employed in different types of proliferating cells by inducing clastogenic or aneugenic damage. It is considered an indirect biomarker of chromosomal damage since at least one cell division is required to visualise MNs. In addition to MNs, several nuclear abnormalities have been described, such as binucleated cells, nuclear buds, lobed and notched nuclei.[41,42] Specifically in amphibians, the analysis of MNs in the circulating erythrocytes of individuals exposed to xenobiotics has become a widely employed technique.[41,42] The advantages of this bioassay include simplicity, low cost and the possibility of evaluating chromosomal instability or mitotic status.[43] Among its limitations, we can include its restriction to cells undergoing the first mitotic division after an injury, performed in nucleated somatic cells, providing an indirect estimate of damage.[35]

12.2.2.2 Single-cell Gel Electrophoresis (SCGE) Assay

The technique is a sensible, rapid, simple and visual methodology employed to provide a direct estimate of damage on single- and double-strand breaks in DNA, alkali-sensitive sites, DNA–DNA and DNA–protein crosslinks, as well as single-strand breaks associated with DNA repair mechanisms.[44–47] SCGE variants have been described, including *in-situ* hybridisation techniques with different fluorochromes (FISH), variable field electrophoresis and the addition of restriction endonucleases within the methodology.[48] The latter is one of the variants adopted worldwide and proposes the inclusion of restriction enzymes, such as Endonuclease III and formamidopyrimidine-DNA glycosylase (FPG), that detect specific DNA lesions.[46] The SCGE assay has become a valuable biomarker in amphibian genetic status evaluation.[37,44–47]

12.2.2.3 Mitotic Index

The cytotoxicity induced by exposure to agrochemicals has also been analysed by employing the mitotic index as a biological endpoint. A gradually decreasing mitotic index value is indicative of toxicity upon division of the cells evaluated. We are aware of only one report that proposes this novel cytogenetic biomarker.[49]

12.2.2.4 Cell Viability

Cell viability is a measure of the proportion of live, healthy cells within a population. Typically, cell viability assays provide a readout of cell health through the measurement of metabolic activity, ATP content or cell proliferation. Its application to Neotropical anurans has been poorly estimated. To the best of our knowledge, Gonçalves *et al.* evaluated sublethal concentrations of the ATZ-based herbicide formulation Atanor 50SC® on cell death of the lesser tree frog *Dendropsophus minutus* tadpoles at different stages of development.[50]

12.2.2.5 Case Study: Herbicides

To the best of our knowledge, there are 23 studies employing cytogenetic biomarkers to detect effects induced by herbicides in Neotropical anurans.[15,38,41,50–53] Moreover, when analysing the data, 47% of these studies have GLY as the evaluated xenobiotic, either as an active ingredient in commercial formulations or in the form of pesticide mixtures. The other 45% is focused on other widely employed herbicides, such as 2,4-D, FLC, imazethapyr (IMZT) and glufosinate ammonium (GLA). In this sense, it is worth mentioning that only two studies were performed evaluating the cytogenetic effects induced by ATZ, the first and second most employed herbicide in Brazil and the Neotropical region, respectively.[15,50]

Considering the type of biomarker used, 65% of the studies employ the MN assay, demonstrating its efficacy for the evaluation of stress induced by agrochemicals on anuran Neotropical species.[40,49,52,54–66] On the other hand, an increase in studies employing the SCGE assay on Neotropical anurans adults and tadpoles has been observed, reaching 56% of the studies performed since 2014.[51] Curiously, this biomarker has not been employed in the region for biomonitoring studies. However, Gonçalves *et al.* demonstrated the importance and efficacy of SCGE in three Neotropical species inhabiting agroecosystem environments.[39] Micronucleous and SCGE assays were performed to evaluate several herbicides as ATZ formulations at sublethal concentrations, detecting cytogenetic damage from 1.5 to 19 mg L^{-1} in *D. minutus* and the Cope's toad *R. diptycha* tadpoles after acute exposure.[15,50] Glufosinate-ammonium and FLC commercial formulations were tested on *R. arenarum* tadpoles, detecting increase in the frequency of MNs to 7.5 mg GLA L^{-1} and 0.71 mg FLC L^{-1} and DNA damage since 0.71 mg FLC L^{-1}.[51,55] Also, when the binary mixture of herbicides was assayed in *R. arenarum* tadpoles, the results showed DNA damage for combinations of GLY-IMZT and GLY-DIC at 5% of $LC_{50\ 96hrs}$ concentrations of each herbicide.[63,64] Added to this, MNs showed an increase in the blacksmith tree frog *Boana faber* and the South American common frog *Leptodactylus latrans* exposed to mixtures of GLY-2,4-D at concentrations of 0.065 mg GLY L^{-1} and 0.004 mg 2,4-D L^{-1}.[66] In addition, the cyto- and genotoxic effects of pure GLY were detected at 0.00125 mg GLY L^{-1} or higher in *D. minutus*.[53] In other Neotropical species, such as the barker frog *Physalaemus cuvieri* and the graceful dwarf frog *P. gracilis*, GLY formulation (Original Roundup Glyphosate®) was shown to produce an increase in MNs starting at 1 mg in chronic exposure.[65] In addition, our working group was the first to use these techniques in combination to evaluate cyto- and genotoxicity in both larvae and adults of the Montevideo tree frog *Boana pulchella*[41,58,59,62] and the oven frog *L. latinasus* exposed to IMZT-based herbicide formulation Pivot® H at environmentally relevant concentrations between intervals of 0.07 and 10 mg IMZT L^{-1}.[60,61] This pioneering work in the region demonstrated the usefulness of using biomarkers in species with different life habits and probable dissimilar contaminants pathways. We have shown that the response of cytogenetic biomarkers in tadpoles and adults is clearly different because they are exposed in a different fashion to agrochemicals. In tadpoles of *L. latinasus,* the tested sublethal concentrations between 0.07 and 0.22 mg IMZT L^{-1} product of a runoff were sufficient to produce an increase in MNs and DNA damage in acute exposure, whereas in adults with a direct application exposure, 10 mg IMZT L^{-1} was necessary to produce DNA damage after 96 h. Finally, we demonstrate the need to apply different cytogenetic biomarkers because they are not only sensitive at different concentrations but also provide different information. Added to this, we applied for the first time the modified SCGE technique and found that *B. pulchella* tadpoles exhibit oxidative damage at 0.39 mg IMZT

L^{-1}.[58] This type of research allowed us to reveal important aspects about the specific mode of action of the herbicide (toxicodynamics), being DNA its target site and, thus, proving that it produces oxidative damage in the DNA of Neotropical tadpoles. This technique has also been successful in assessing oxidative damage in DNA from *R. arenarum* adults exposed to effective concentrations (20 mg L^{-1}) of GLY and 2,4-D. To conclude this section, although works with modified SCGE are incipient, it has been demonstrated that they are important biomarkers for understanding the unknown effects of agrochemicals.

12.2.2.6 Case Study: Insecticides

To the best of our knowledge, only 15 studies employing this biomarker in Neotropical anurans have been reported so far.[34,38,40,42,67–76] Of these studies, approximately 35%, 20%, and 13% apply cytogenetic biomarkers in OP insecticides, in both NEO and PYR (including fourth-generation PYR) and in carbamates, respectively. Approximately 6% corroborated its response when OC were assayed. It should be mentioned that the most widely used cytogenetic biomarkers in Neotropical anurans for the evaluation of insecticides are MNs, representing almost 67% of the studies. Regarding SCGE, in the Neotropical region, only two studies have used this indicator of direct DNA damage in a modern insecticide such as imidacloprid (IMI) in commercial formulation and active compound.[42,76] New studies in Neotropical anurans should address the use of these globally validated biomarkers for studies on insecticides, as they provide greater sensitivity than the most widely used MN assay. Among insecticides, IMI was evaluated in *B. pulchella,* which produces genotoxicity in acute exposures.[42,76] Chronic exposures in the South American spotted grass frog *L. luctator* and the Barker frog *P. cuvieri* tadpoles[74] produce MNs that increase at 0.1 mg IMI L^{-1}. Added to this, *B. pulchella* tadpoles showed an increase in MNs following exposure to 0.005 mg endosulfan L^{-1} and 58.52 mg pirimicarb L^{-1} after 96 h of exposure.[67,72] For pirimicarb, it is important to note that acute exposure between 80 and 160 mg L^{-1} produces significant increases in MNs in *R. arenarum* tadpoles.[70] Similarly, cypermethrin showed an increase in MNs in *O. asper* (= *O. americanus*) tadpoles after acute exposure to 0.005 mg L^{-1},[68] and the organophosphorate chlorpyrifos (CPY) increased MNs in Carvalho's escuerzo *O. carvalhoi* tadpoles (at a relevant concentration of 0.1 mg L^{-1})[73] and induce oxidative pyrimidine damage in *R. arenarum* tadpoles (acute exposure of 0.01 mg L^{-1}).[40] These works represent evidence that cytogenetic biomarkers as well as mortality can be used to assess the response of Neotropical anurans to insecticides.[68,73,77] It should be noted that our studies in *B. pulchella* not only were the first to combine both cytogenetic methodologies but also revealed that the SCGE assay is more sensitive than MNs in detecting DNA damage at early exposure times and at low concentrations of the agrochemical of interest, in this case between 30 and 37 mg IMI L^{-1}.

12.2.2.7 Case Study: Fungicides

Studies employing cytogenetic biomarkers in fungicides are scarce. Only Asis *et al.* found cytotoxic effects in *L. luctator* tadpoles induced by the fungicide formulation Elatus® containing a mixture of azoxistrobin and benzovindiflupyr at relevant environmental concentrations ranging from 0.01 to 0.05 mg L^{-1} during 96 h.[78] It is important to mention that only MN analysis was carried out in conjunction with the evaluation of other nuclear abnormalities, demonstrating the usefulness of the bioassay and the wide variety of exposure scenarios for this biomarker. However, it would be important to expand studies on fungicides to evaluate the response of native anurans, as they are the third most used pesticide in the Neotropical region.

It is noteworthy that Neotropical anurans are valid models for evaluating the agrochemical impact and that the MN and SCGE assays have been probed as useful tools. Although the aforementioned techniques have shown that they can be employed as valid endpoints for detecting pesticide-induced deleterious effects in Neotropical native anurans, it would still be important to focus on studies that try to find out how species not employed as biological matrices so far respond to these agents.

12.2.2.8 Case Study: In Situ Biomonitoring Studies

In addition, field studies at agrochemical sites have used MN and SCGE assays as biomarkers in tadpoles of *B. albopunctatus,*[39] *D. minutus*,[39,79,80] *R. arenarum,*[81] the San Luis snouted tree frog *Scinax fuscovarius*[39,80] and adults of *L. luctator*.[82] As previously mentioned, MNs are the most widely used and validated cytogenetic biomarkers in field evaluations.[81–87] Once again, it should be noted that recent studies have proposed potential novel cyto- and genotoxicity biomarkers in Neotropical anurans, such as the TUNEL assay, to evaluate the induction of apoptotic cells,[69] the frequency of erythroblasts,[72] the H/L ratio[40] or even the expression of c-Fos y Mek genes on Neotropical anurans embryos.[71]

12.2.3 Biochemical Biomarkers

Understanding biochemical mechanisms allows us to predict the effects of several unknown environmental stressors based upon their similarity in biochemical mode of action to well-known pollutants.[3] Specifically for anuran, enzymatic variations related to oxidative stress and cholinergic pathways are the most employed biochemical biomarkers. Agrochemicals are generally linked to oxidative stress mediated directly by the generation of reactive oxygen species (ROS).[3,4] Changes in these enzymes might be observed in individuals at contaminated sites or those exposed to stressors under laboratory conditions.[3] Besides, the measurement of antioxidant enzymes could be used as a biomarker of oxidative stress.[3,13] Another point to consider is the enhancement in enzyme concentration and/or activity related to the developmental stage of the specimens.[8] Among the enzymes commonly involved, we can name catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione *S*-transferase (GST) and 7-ethoxyresorufin-*O*-deethylase (EROD) as enzymatic antioxidant biomarkers. On the other hand, reduced glutathione (GSH) and lipid peroxidation (TBARS or LPO) can be grouped as non-enzymatic antioxidant biomarkers. Finally, acetylcholinesterase (AChE), butyrylcholinesterase (BChE) and carboxylesterases (CabE) are grouped as cholinergic-stress enzyme biomarkers.

12.2.3.1 Case Study: Herbicides

To the best of our knowledge, 15 works employing tadpoles or adults are reported for the evaluation of the effects of herbicides.[25,49,52,54,60–62,88–96] So far, biochemical biomarker responses have been most tested in the herbicide GLY, in approximately 55% of the cases. The trend indicates that this class of biomarkers has been extensively evaluated in IMZT and GLA in around 20% of the studies. Focusing on enzymes, the most employed biomarkers were ChE and GST in 87% of the studies. Interestingly, enzymes directly related to oxidative stress (*e.g.,* LPO, CAT and SOD) have been poorly studied; only 45% of the cases have attempted to envisage the ROS potential of herbicides. Unfortunately, no studies are evaluating the response of EROD to Neotropical anuran post-agrochemical exposures. In particular, decreases in the activities of GST, ChE and CbE have been reported in different commercial formulations of GLY (ranging from 2 to 120 mg GLY L^{-1}),[89] metsulfuron-methyl, bispyribac-sodium and PCM (ranging from 0.0097 to 160 mg herbicide L^{-1}) after acute exposure in *R. arenarum* tadpoles.[54] In other studies, the commercial herbicide-based CMZ formulation (Gamit® 360CS) showed alterations in biochemical antioxidant biomarkers by an increase in GST, CAT, SOD and G6PDH activity when the Cuyaba dwarf frog *Eupemphix nattereri* and *R. diptycha* tadpoles were exposed in acute bioassays to concentrations ranging between 0.01 and 0.1 mg L^{-1}.[93] Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) were inhibited when GA was evaluated in *B. pulchella* tadpoles after acute exposure of 3.5 to 15 mg L^{-1} of the commercial formulation Liberty®,[92] coupled with an increase of GST activity reported at 20 mg of 2,4-D L^{-1}.[40] IMZT was evaluated in two species with different life modes (*B. pulchella* and *L. latinasus*) and in their two life stages (tadpoles and adults).[60–62] If we focus on these results obtained by our group, we can corroborate the importance of using a battery of biochemical biomarkers according to the exposure situation, the species in question, and the life stage of the Neotropical anurans. In this case, we demonstrate that antioxidant enzyme responses vary according to anuran life stages when exposed to the same agrochemical. To note, in *L. latinasus* tadpoles, it was necessary to use a sublethal concentration of 0.15 mg L^{-1} to induce an increase in GST activity;

for *L. latinasus* adults, it was necessary to use real acute exposure scenarios of 1000 mg IMZT L^{-1} to trigger increases in AChE and 10 mg IMZT L^{-1} to trigger the antioxidant response with concomitant CAT increase and inhibition of GST. In contrast, *B. pulchella* adults showed CAT inhibition and increased GST antioxidant response in acute exposure scenarios at 10 and 100 mg IMZT L^{-1}, although it is noteworthy that AChE showed the same response as *L. latinasus* for the same scenario assayed.

12.2.3.2 Case Study: Insecticides

To the best of our knowledge, 27 works employing Neotropical anurans reported evaluations on the effects of insecticides in pure formulations or mixtures of active ingredients on biochemical biomarkers.[40,71,75,95,97–116] In Neotropical species, the most widely assayed insecticides are OP, with approximately 85% of the studies and only 8% on carbamates and 1% on OC. Among insecticides in *R. arenarum* tadpoles, pure malathion[100–102] induces increases in AChE and CAT and decreases in TBARS and GR at concentrations between 4 and 20 mg L^{-1} in both chronic and acute bioassays; pure azinphos-methyl[99,103,108,109] and carbaryl[99,103] induce inhibition of AChE, CabE and antioxidant responses of GSH, CAT and SOD, while an increase was observed in GST and GR at concentrations between 0.2 and 20 mg L^{-1} in chronic *in situ* or acute *ex situ* exposure. Pure and commercial formulations of OPE insecticide CPY, at environmentally relevant concentrations, in acute and chronic exposures, induce inhibition of GST, ChE and CabE enzymes and an increase in anti-ROS production enzymes such as GSH and CAT in tadpoles.[71,110,111] Evaluations in adults induce inhibition of BChE and CabE and an increase in CAT.[105] Also, the commercial formulation PYR-Trisada®, containing a mixture of the synthetic PYR insecticides deltamethrin and tetramethrin, was evaluated in acute exposures.[106] CabE and AChE inhibition were detected at a concentration range of 0.0003125–0.00125% in *R. arenarum* tadpoles. Other studies were performed employing novel species, such as *X. laevis* for the evaluation of OPE insecticide fenitrothion-based formulation in a recovery assay to sublethal concentrations (0.5 to 1.5 mg L^{-1}) that caused inhibition of BChE and AChE,[104] *S. fuscovarius* for the evaluation of pure diazinon in acute exposure to sublethal concentrations (1 to 3 mg L^{-1}) that caused inhibition of CbE and AChE.[107] Chlorpyrifos induced reduction in AChE and CabE activity and GST increase in tadpoles of *B. pulchella* to environmentally relevant concentrations (0.05 to 5 mg CPY L^{-1}).[113] *Physalaemus gracilis* was also evaluated with CPY, which induces AChE inhibition and anti-ROS production enzyme (SOD and GST) increase at relevant concentrations of 0.9 mg L^{-1} of the commercial formulation Klorpan 480EC.[97] Cypermethrin induces AChE, BChE and CAT inhibition, and SOD and GST increase at concentrations of 0.006 mg L^{-1} of the commercial formulation Cyptrin 250CE.[98] Fipronil induces AChE, BChE and SOD inhibition, and anti-ROS enzymes (CAT and GST) increase at relevant concentrations of 0.026 mg L^{-1}

at the commercial formulation Terra Forte®,[86] and the same insecticide in its pure ingredient promotes G6PDH, CAT increase and MDA inhibition in *E. nattereri* at doses starting at 0.035 mg kg^{-1}.[112] Finally, some authors have begun to test these biomarkers in new insecticides of natural origin, such as *Bacillus thurigensis*,[91] derivatives of natural origin, such as pyriproxyfen,[56] and spinosad[117] or even in molluscicides.[118]

12.2.3.3 Case Study: Fungicides

To the best of our knowledge, only one study has been performed at the time of publication of this book to evaluate the acute and chronic effects of pure broad-spectrum fungicide chlorothalonil on biochemical biomarkers using the red-eyed tree frog *Agalychnis callidryas*, the meadow tree frog *Isthmohyla pseudopuma* and the common Mexican tree frog *Smilisca baudinii* tadpoles from Costa Rica at concentrations ranging from 0.0025 to 0.1 mg L^{-1}.[119] In this case, at higher concentrations, an increase in muscle ChE activity was detected in *I. pseudopuma* and the liver GST activity increased in *S. baudinii*.[119]

12.2.3.4 Case Study: Biomonitoring In Situ Studies

For Neotropical anurans that live exposed to agrochemicals, studies are focused on biomonitoring or mesocosm. The biochemical biomarker mostly used in all reports published is AChE, followed by GST in 90% and CAT in 65% of cases. Specifically, several biomonitoring studies were performed on adults of *R. diptycha*,[120] *L. chaquensis*,[121] *B. pulchella*,[122,123] *R. arenarum*,[122] the Uruguayan harlequin frog *Lysapsus limellium*,[124] *L. luctator* and *L. latinasus*[125] that report alterations in the most commonly used enzymes, such as ChEs, CbE, GR, GST and CAT. *Rhinella arenarum* tadpoles have been employed in bioassays to evaluate biochemical biomarkers in sediments containing agrochemicals using different enzymes, such as esterases and antioxidants, as effective biomarkers.[81] Recently, a mesocosm study with tadpoles of *E. nattereri* and *S. fuscovarius* exposed to agrochemicals in the field reported adverse responses using the same set of biomarkers.[126] Finally, AChE and GST showed an increase in the Rufous frog *L. mystacinus* tadpoles, but BChE, AChE and GST decreased their activity in the striped snouted tree frog *S. squalirostris* tadpoles from agricultural areas.[34]

For this class of biomarkers, the wide variety of enzymes commonly used to evaluate agrochemicals in Neotropical anurans is worth noting. This situation has allowed us to learn more about which agrochemicals induce ROS and the enzymatic mechanisms required to counteract them.

12.2.4 Reproductive Biomarkers at the Physiological, Biochemical and Genetic Levels

Reproductive biomarkers are measurable changes that directly or indirectly affect reproductive success and, in turn, involve a series of responses stemming from the molecular level up to the highest biological organisation scale (see Figure 12.1). This results in an integrative tool that links the consequences of reproductive success on populations in anthropogenically disturbed ecosystems.[3,4,13] As most anuran species have their reproductive peak in spring, in many cases coinciding with the period of application of many agrochemicals, they would be especially affected by agents during the reproduction phase, either by direct application or by runoff after intense rains.[127]

Reproductive biomarkers can be evaluated at physiological and biochemical levels in response to agrochemicals that mimic endogenous molecules that produce hormone alterations and endocrine disruption, mainly in the hypothalamic-pituitary-gonadal axis. Furthermore, it has also been shown that other metabolic alterations could indirectly affect amphibian reproduction.[29,127] EDCs' main effects linked to reproduction can be oestrogenic, anti-oestrogenic, androgenic, anti-androgenic and progestogenic.[13,29] These effects can be evidenced, for example, through circulating levels of sex steroids,[128,129] induction of hepatic biosynthesis of vitellogenin,[128,129] alterations on gametogenesis through gonadal histology,[130] or simply by gonad size (*e.g.*, gonadosomatic index).[121,123] There are also differences in the expression of genes related to sexual cycles in adults or sexual differentiation in anuran tadpoles.[131,132] In addition, perturbations of reproductive behaviour (*e.g.,* calling in males) can be considered a reproductive EDC biomarker,[133,134] including alterations in secondary male characteristics such as nuptial pads that become greater and blackish in the breeding season.[133–135] Another commonly evaluated endpoint in anurans is fertility. The indirect measurement of fertility can be done by counting viable gametes in the gonads, evaluating both sperm morphology and the number of viable eggs.[136,137] If DNA damages induced by agrochemicals are produced in germinal cells, they become inheritable and are passed on to the next generation.[3,13] To the best of our knowledge, there are no known studies evaluating the genotoxic effects on germ cells of agrochemical-exposed Neotropical anurans.

12.2.4.1 Case Studies: Insecticides

To date, there are no reports evaluating reproductive biomarkers in Neotropical anurans exposed to insecticides. Recently, genotoxic effects have been found in germ cells of adult *R. arenarum* males exposed to the NEO insecticide IMI in realistic exposure scenarios using the sperm SCGE assay (Bach and Cid, unpublished data; see Figure 12.3). The aforementioned study also included the first biochemical evaluations in sperm

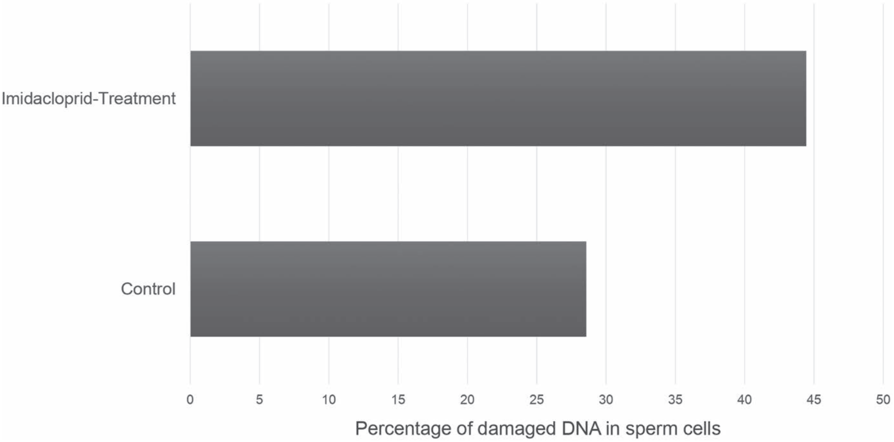

Figure 12.3 DNA damage evaluated by the single-cell gel electrophoresis (SCGE) assay in spermatozoa from the South American common toad *Rhinella arenarum* exposed to the neonicotinoid insecticide imidacloprid.

using CAT and LPO as biomarkers, which demonstrated oxidative stress in anuran germinal cells after IMI exposure.

12.2.4.2 Biomonitoring In Situ Studies

Alterations in spermatogenesis have only been reported in histological analyses of several cell types in the testes of adult individuals of *R. fernandezae*, the Sanborn's tree frog *D. sanborni*, *L. limellum*, the pigmy toad *R. bergi*, *P. cuvieri*, *D. minutus* and *B. albopunctata* that inhabit different ecosystems in which the presence of agrochemicals has not been determined yet.[90,138,139]

12.3 Perspectives

12.3.1 Trends in Neotropical Anurans

We have observed a significant increase in the number of studies using biomarkers for agrochemical evaluations in Neotropical anurans during the last twenty years (see Figures 12.4A and 12.4B). These studies show the usefulness of deploying a battery of various biomarkers in different agrochemical exposure scenarios. In herbicides, most of the studies are focused on the use of biomarkers for GLY; therefore, new studies should focus more on applying biomarkers to evaluate the effects of new herbicides. On the contrary, studies on insecticides have been focused on accompanying the current agricultural model (*e.g.,* NEO). Unfortunately, studies with fungicides using biomarkers are scarce. In this sense, further

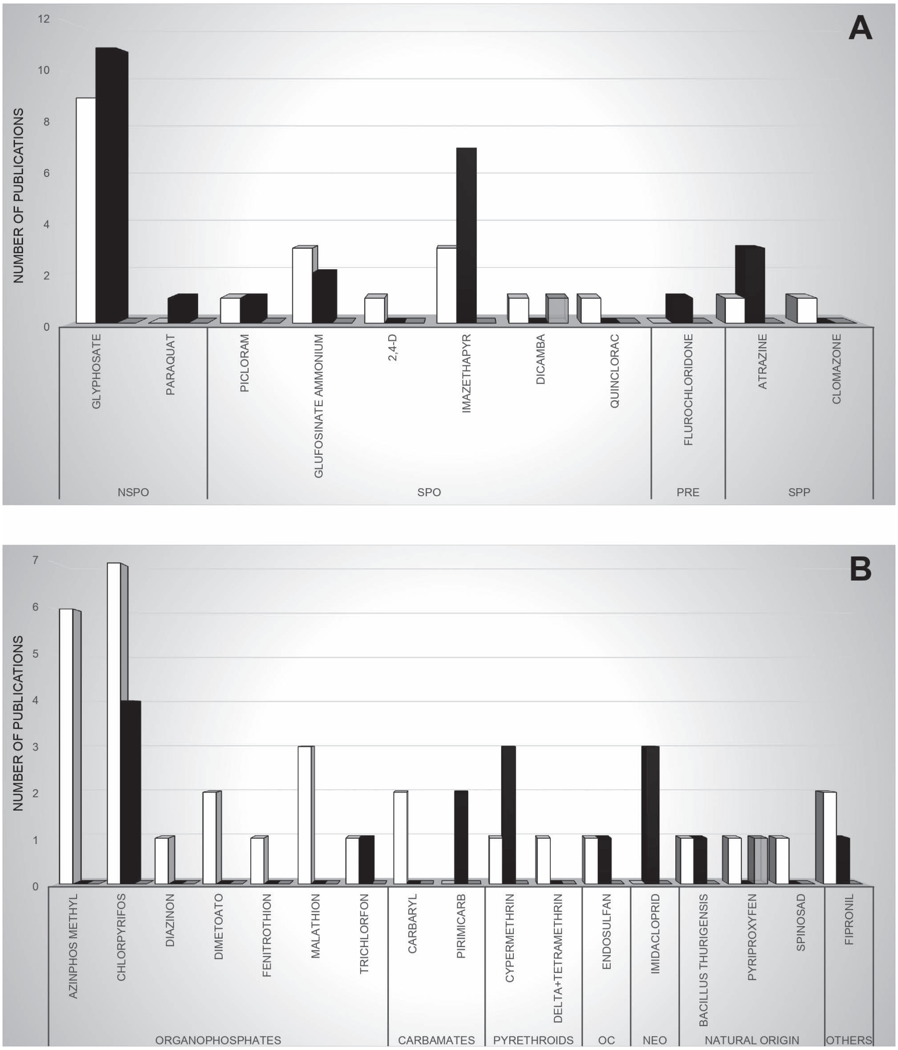

Figure 12.4 Different types of biomarkers used in Neotropical anurans to assess the effects of herbicides (A) and insecticides (B). White bars represent biochemical, black bars cytogenetic and grey bars physiological biomarkers. NSPO, non-selective post-emergent; SPO, systemic post-emergent; SPRE, selective pre-emergent; SPP, selective pre- and post-emergent herbicides; OC, organochlorines; NEO, neonicotinoids; OP, organophosphate insecticides.

studies are necessary to determine whether these biomarkers are suitable for fungicide risk assessment in Neotropical anurans or if new biomarkers should be pursued. To be factored into the drawing of conclusions, it is important to note the present lack of knowledge about the effects of agrochemicals on the physiological biomarkers of Neotropical anurans. Having said this, the future use of biomarkers in Neotropical anurans and risk assessment strategies is promising, both in bioassays with agrochemicals and in biomonitoring studies in agricultural regions. Finally, it is important to note that some terms used, such as cytogenetics and genotoxicity, do not always refer to the same biomarkers, and it would be important to review their generalised use to avoid confusion and speak a common language.

12.4 Anuran Models

The studies previously mentioned show that the most employed species for evaluating the effects induced by agrochemicals is *R. arenarum,* a terrestrial species. However, there is an increasing trend of studies using *B. pulchella* and *S. nasicus* (arboreal species), *L. luctator*, *O. laevis, P. cuvieri* and *P. gracilis* (semi-aquatic species), *L. latinasus, E. nattereri* (cavicolous species) or *R. diptycha* (terrestrial species) (see Figure 12.5). This approach of employing species with different habitats would allow for a better understanding of the effect of agrochemicals on native biota.

Most studies were performed employing species belonging to the Pampas region of Argentina (see Figure 12.6). It is also worthwhile and necessary to consider studies in species that inhabit other regions where the use of agrochemicals is also intensive, *e.g.*, the Great South American

Figure 12.5 Main Neotropical adult anurans proposed as models for risk assessment studies after agrochemical exposure. Images depict *Boana pulchella* (A), *Leptodactylus latinasus* (B), *Odontophrynus laevis* (= *O. americanus*) (C) and *L. luctator* (D).

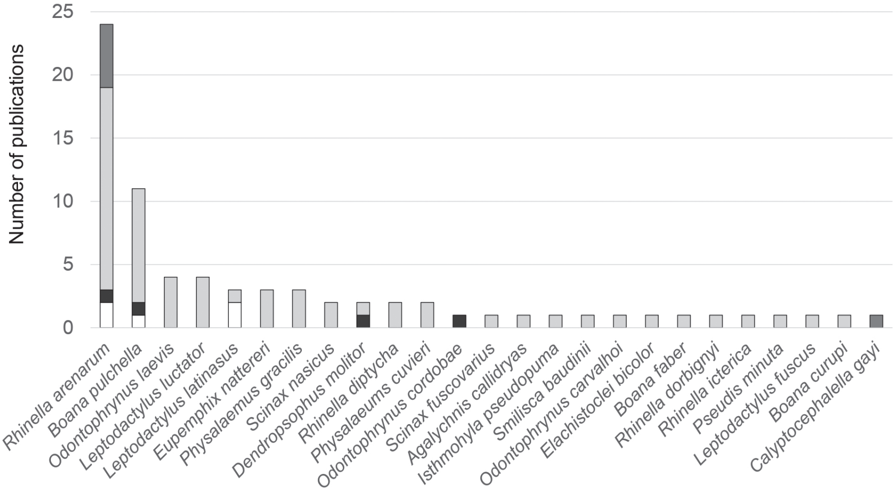

Figure 12.6 Neotropical anuran life stages employed as models for risk assessment studies after agrochemical exposure. Bars represent: adults (white), metamorphs (black), tadpoles (light grey) and embryos (dark grey).

Chaco shared by Argentina, Bolivia, Brazil and Paraguay. It would also be a *sine qua non* requirement to establish unified protocols for the biomarker assessment to be able to compare results from different laboratories, as the techniques employed generally differ between species and life stages of Neotropical anurans. Finally, as we emphasised in our work with IMZT, it is necessary to evaluate agrochemicals in both tadpoles and adults to obtain results that are more representative of what is happening in the region.

12.5 Lack of Linking Biomarkers: A Multibiomarker Approach

Biomarkers are sensitive tools that indicate that environmental stressors have entered an organism and have been distributed among tissues, in turn causing detrimental effects.[3,15] The comprehensive application of a battery of biomarkers could improve the interpretation of the effects induced by agrochemicals and assist in environmental risk assessment, management and the decision-making process before irreversible damage occurs in anuran populations or, even worse, in Neotropical ecosystems.[3,4,8,13] In this context, the "multibiomarker approach" to evaluate and biomonitor the environmental quality of water and soil is recommended by modern ecotoxicology/toxicology. A long pathway has yet to be travelled to arrive at this point, as previously discussed.[3,13,15,60–62] Our works have incorporated the current most worldwide employed approaches and, for the first time, tried to integrate several biomarkers at different ecotoxicological levels (*e.g.*, individual, biochemical and cytogenetical). The use of both life stages of Neotropical anurans (*e.g., B. pulchella* and *L. latinasus*) exposed to a novel herbicide, such as IMZT at relevant environmental concentrations, helps us in our endeavours to seek explanations that biomarkers alone do not provide and/or make for a more realistic risk assessment. This demonstrates that a holistic and integrative view is an important tool required for this task.[60–62] Our work clearly showed that depending on the xenobiotic concentration, a different biomarker should also be called in for a complete agrochemical biomonitoring programme since responses in Neotropical anuran species are different *vis-à-vis* the same agent(s). This situation would allow us to not only predict the presence of such environmental stressors but also enable us to take the necessary steps and actions to avoid irreversible effects. Undoubtedly, future work with Neotropical anurans and agrochemicals should consider this approach to improve the analysis and refine results further. This, in turn, would allow for the drafting of regulations that would ensure the responsible use of these chemicals in the environment.

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