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REVIEW
Biomarkers of effect in toads and frogs

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Amphibians are good bioindicators of environmental pollution due to their susceptibility to chemicals during their freshwater cycles. The effects of environmental pollution, together with changes in human activity and climate, have contributed to the reduction in the amphibian population over recent decades. However, toxicological research on amphibians has been rather scarce compared with that on other vertebrates. In this article we review the biochemical alterations underlying xenobiotic action and/or the detoxifying responses described for anuran species, with the aim of establishing possible biomarkers of effect. During the embryonic development of anurans, morphological and behavioural alterations are the effects most frequently cited in connection with chemical exposures. However, such biomarkers have a low sensitivity and are unspecific compared with biochemical alterations. Some primary pesticide targets, in particular cholinesterases for organophosphates and carbamates, have been evaluated. Esterases change seasonally and with the stage of development, and their sensitivity to anticholinesterase agents varies between species. Thus their use as biomarkers in anurans must be carefully analysed. Enzymes and endogenous compounds related to oxidative metabolism may also be used as biomarkers of effect. Glutathione pool, glutathione-*S*-transferases and metallothioneins respond in different ways to pesticides and heavy metals in anuran embryos and tadpoles. Mixed-function oxidases, in turn, are less developed in amphibians, and show a reduced induction in response to pesticide exposures. Endogenous polyamine levels are also proposed as good age-related biomarkers of damage. Finally, molecular biomarkers related to receptor binding, signal transduction and genetic response have gained increasing relevance, as they have been implicated in the fertilisation process and the earliest events in anuran development. The identification of transcription factors associated with the exposure of amphibians to xenobiotics as well as other alterations in hormone signalling appears highly promising. However, these techniques are likely to complement other methods. In conclusion, the use of several biomarkers with multiple endpoints is needed to link exposure to response and to provide better predictive tools for the environmental protection of endangered anuran species.

Keywords: amphibian, anuran, teratogens, cholinesterases, oxidative stress, molecular biomarkers.

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Ecotoxicology of amphibians and the decline of global populations: the need for biomarkers

Amphibian populations have been declining globally over recent decades (Barinaga 1990, Wake 1991, Houlahan *et al.* 2000). The decline in South-American species has been even faster than the global mean, independently of a single worldwide (Houlahan *et al*. 2001) or a spatially and temporarily varying trend (Alford *et al*. 2001). Several reasons have been put forward to explain such declines, some arising directly or indirectly from human activities and others emerging from global and local climatic changes. These include the following: direct destruction of amphibian habitats by humans, chemical pollution, acid rain, fungal and bacterial infection, which may be in turn related to ozone depletion and an increase in ultraviolet exposure (Blaustein *et al*. 1994) and relative drought seasons in high altitude sites related to El Niño/Southern Oscillation cycles (Klesecker *et al*. 2001).

The ecotoxicology of amphibians has received scarce attention compared with that of other vertebrates. Nevertheless, a complete review on the topic has been recently published (Sparling *et al*. 2000). The authors report that only 2.7% of the literature covering aspects of ecotoxicology in the last 25 years (up to 1998) concerned amphibians. The reasons for this disparity are not clear, since the biomass of amphibians, their importance in the trophic chain and their relevance in the loss of biodiversity justify ecotoxicological concern. Amphibians are found in quite different habitats such as desert and forest, in varying climates and different altitudes. Because amphibians pass their first life stages in water and their adult life span as terrestrials, they are exposed to a wide range of contaminants. This, together with their feeding habits, the continuous processing of water through their gills, and their particular sensitivity to chemicals during their freshwater cycles, makes amphibians good bioindicators of the overall conditions of the environment (Dumpert and Zeitz 1984, Lefcort *et al*. 1998). Most amphibian species are susceptible to xenobiotics during fertilization and early development (Cooke 1972, Devillers and Exbrayat 1992).

Over the last decade, biomonitoring has been increasing used to track environmental pollution (Whitfield 2001). Chemical analysis of water and soils represents a direct proof of the nature and degree of contamination, but sensor organisms can reveal the status of the dynamic scenario. Biomarkers of exposure, effect and susceptibility are needed to connect the presence of pollutants in the environment with their action in an organism. In this context, biomarkers can aid in assessing the health status of amphibian populations by acting as sublethal endpoints of intoxication.

We have been studying the effects and mechanisms of action of pesticides in the embryonic and larval ontogenesis of the South American toad *Bufo arenarum*, evaluating acute and chronic toxicology on recognized primary as well as secondary molecular targets. In the following section we review the biochemical alterations underlying xenobiotic action and/or the detoxifying responses described for anuran species, discussing their use as possible biomarkers of effect in amphibians in general and *Bufo arenarum* in particular, mainly in the embryonic and larval stages.

Morphological, physiological and biochemical changes caused by xenobiotics in anurans as biomarkers of effect

Morphological alterations induced in embryogenesis

Morphological alterations and impairment of normal growth are the most frequently described biomarkers of exposure to pesticides and other contaminants such as heavy metals. Table 1 summarizes the main developmental alterations described in the literature for anurans. Early work by Kaplan and Overpeck (1964) and Cooke (1970, 1972) described hyperactivity in frog and toad tadpoles exposed to organochlorine (OC) pesticides. We have evaluated the malformations elicited by OCs during the embryonic development of *Bufo arenarum*. Exposures were in general characterized by the appearance of body twisting and progressive dropsy, notocord alteration, defective gills, reduced weight and size, and shortening of the time to reach metamorphosis (De Llamas *et al*. 1985, Gauna *et al*. 1991, Caballero de Castro *et al*. 1997, Anguiano *et al*. 2001). These alterations have also been described in other exposed species (Schuytema *et al*. 1991). Organophosphates (OPs) and carbamates also cause body shortening with caudal fin folding, notocord bending and abnormal pigmentation in *Bufo arenarum* (Rosenbaum *et al*. 1988, Anguiano *et al*. 2001). Impaired growth, reduced body size, haemorrhage, abnormal organogenesis and paralysis have been described in other OP-exposed anuran embryos and tadpoles (Snawder and Chambers 1989, Alvarez *et al*. 1995, Schuytema *et al*. 1995, Berrill *et al*. 1997, Harris *et al*. 1998, Fordham *et al*. 2001). Heavy metals affect notocord, head, gill and caudal fin formation in *Bufo arenarum*, causing a decrease in growth and size and delaying development (Pérez-Coll *et al*. 1985, 1988). Similar alterations were observed in *Rana catesbeiana*, *Rana luteiventris* and *Xenopus laevis* (Rowe *et al*. 1996, Herkovits *et al*. 1997, Lefcort *et al*. 1998). Polychlorinated biphenyls (PCBs), pyrethroids, herbicides, fungicides and inclusive ultraviolet radiation are able to produce some of these developmental alterations in different anuran species (Table 1).

It would seem that the alterations in morphology and development are not specific for any class of compound. Most of the alterations are encountered after gastrulation has been completed. Table 2 summarizes the different effects described for the different classes of compounds in Table 1. The time needed for metamorphosis is increased for nearly all types of compounds, except for some OCs, which may produce a shortening of the development period. Both tadpole weight and size are commonly reduced by xenobiotics. The larval body is generally curved by chemical exposure, presenting a bent notocord, with defective formation of gills, gut and caudal fin, and generalized oedema. Haemorrhage was only described with OP exposure. Abnormal pigmentation in tadpoles is also a defect common to various classes of xenobiotics. The behavioural alterations caused by pesticide target impact include paralysis-hyperexcitability, body twisting, spasms, convulsions, limited movement and altered swimming.

Amphibians are massive reproducers that frequently display a higher number of malformations in normal populations (about 1%) than other species (Crawshaw 2000). Thus, distinguishing malformations due to xenobiotic exposure in the field from the normal background level may be rather difficult. Malformations have been detected in a few studies using caged tadpoles exposed to OCs, OPs, carbamates

Table 1. Anatomical and physiological alterations in growth induced by xenobiotics and physical agents during anuran development.

Compound	Species and stage	Effect	Reference
OCs			
Aldrin	*Rana pipiens*	Hyperactivity	Kaplan and Overpeck 1964
Chlordane	*Rana pipiens*	Hyperactivity	Kaplan and Overpeck 1964
DDT	*Bufo arenarum* larva	Body twisting; defective gills; progressive dropsy; reduced weight; shortening of metamorphosis time; erratic swimming; hyperexcitability	Caballero de Castro *et al*. 1997
	Rana temporaria tadpole	Reduced weight; notocord alteration; deformed snout; hyperactivity	Cooke 1970, 1972
	Rana sylvatica embryo	Hyperactivity	Licht 1985
Dieldrin	*Bufo arenarum* embryo	Exogastrulation; arrest in gastrula	Anguiano *et al*. 2001
	Bufo arenarum larva	Reduced size; abnormal pigmentation; defective gills; progressive dropsy; shortening of metamorphosis time	De Llamas *et al*. 1985
		Tail lashing; body twisting; erratic swimming; hyperexcitability	Gauna *et al*. 1991; Caballero de Castro *et al*. 1997
	Bufo bufo; Rana temporaria	Hyperactivity	Cooke 1970, 1972
	Rana pipiens	Hyperactivity	Kaplan and Overpeck 1964
	Xenopus laevis; Rana catesbeiana	Notocord deformity	Schuytema *et al*. 1991
Endosulfan	*Rana sylvatica* tadpole; *Rana clamitans* tadpole	Paralysis (low levels), hyperactivity (high levels)	Berrill *et al*. 1998
	Bufo americanus tadpole	Also impaired metamorphosis	Berrill *et al*. 1998
Endrin	*Rana sphenocephala* tadpole	Hyperactivity	Hall and Swineford 1980
	Bufo americanus; Rana catesbeiana; Rana sylvatica	Erratic swimming; disturbed equilibrium; abnormal posture	Hall and Swineford 1981
Lindane	*Bufo arenarum* embryo	Arrest in gastrula	Anguiano *et al*. 2001
	Bufo arenarum larva	Body twisting; defective gills; progressive dropsy; reduced weight; shortening of metamorphosis time; erratic swimming	Caballero de Castro *et al*. 1997
		Caudal fin bending; hyperactivity	Anguiano *et al*. 2001
Toxaphene	*Rana pipiens*	Hyperactivity	Kaplan and Overpeck 1964
	Rana sphenocephala tadpole	Hyperactivity	Hall and Swineford 1980
	Rana catesbeiana; Rana sylvatica; Bufo americanus	Erratic swimming; disturbed equilibrium; abnormal posture	Hall and Swineford 1981

Table 1 (*Continued*)

Compound	Species and stage	Effect	Reference
PCBs			
Arochlors	*Bufo americanus; Bufo fowleri*	Lordosis; scoliosis; oedema	Eisler and Beslisle 1996
Clophen; PCB 126	*Xenopus laevis; Rana temporaria*	Oedema; depigmentation; tail deformity; increased weight; delayed metamorphosis	Gutleb *et al*. 2000
OPs			
Azinphos methyl	*Bufo arenarum* larva	Reduced size; notocord bending; abnormal pigmentation; defective gut and gills; swimming in circles	Caballero de Castro *et al*. 1997
	Rana clamitans embryo	Body shortening	Harris *et al*. 1998
	Hyla regilla tadpole; *Xenopus laevis* tadpole	Impaired growth	Schuytema *et al*. 1995
Fenitrothion	*Bufo americanus* tadpole; *Rana catesbeiana* tadpole; *Rana clamitans* tadpole; *Rana pipiens* tadpole; *Rana sylvatica* tadpole	Paralysis	Berrill *et al*. 1997
Malathion	*Bufo arenarum* larva	Body twisting; tail lashing; limited movement	De Llamas *et al*. 1985
		Reduced size; tail folding; notocord bending; oedema	Rosenbaum *et al*. 1988
		Haemorrhage; reduced pigmentation; defective gut	Caballero de Castro *et al*. 1997
		Reduced gills; swimming in circles	Anguiano *et al*. 2001
	Rana catesbeiana tadpole	Abnormal gills; haemorrhage; altered equilibrium; paralysis	Fordham *et al*. 2001
(also malaoxon)	*Xenopus laevis* embryo	Reduced size; abnormal pigmentation; abnormal gut; notocord bending	Snawder and Chambers 1989
Methyl parathion	*Rana perezi* larva	Notocord bending; scoliosis; tail folding	Alvarez *et al*. 1995
Parathion	*Bufo arenarum* larva	Reduced size; notocord bending; oedema; haemorrhage	Caballero de Castro *et al*. 1997
		Reduced pigmentation; defective gut and gills; swimming in circles	Anguiano *et al*. 2001

Table 1 (*Continued*)

Compound	Species and stage	Effect	Reference
Carbamates			
Carbaryl	*Bufo arenarum* larva	Reduced size; notocord bending; abnormal pigmentation; defective gut and gills; swimming in circles	Caballero de Castro *et al*. 1997
	Rana tigrina tadpole	Reduced weight and growth	Marian *et al*. 1983
	Rana blairi tadpole	Reduced motility	Bridges 1997
Carbofuran	*Microhyla ornata* tadpole	Tail bending; abnormal swimming	Pawar and Katdare 1984
Oxamyl	*Rana temporaria* tadpole	Body twisting; tail tip; malformations reduced development	Cooke 1981
Pirimicarb	*Rana perezi* tadpole	Notocord bending; limb malformation	Alvarez *et al*. 1995
Propoxur	*Rana hexadactyla* tadpole	Tail kinking; increased length; limb malformation	Raj *et al*. 1988
Pyrethroids			
Esfenvalerate	*Rana blairi* larva; *Rana sphenocephala* larva	Spasm; convulsive twisting; weight loss	Materna *et al.* 1995
Fenvalerate; permethrin	*Rana clamitans* embryo and larva	Slower growth; body bending	Berrill *et al*. 1993
	Rana pipiens embryo and larva	Paralysis; weakness	Berrill *et al*. 1997
	Rana sylvatica embryo and larva; *Bufo americanus* embryo and larva	Delayed metamorphosis	Berrill *et al*. 1993
Herbicides			
Cyanatryn	*Rana temporaria* tadpole	Lethargy; spasm; reduced weight	Scorgie and Cooke 1979
Diuron	*Hyla regilla; Xenopus laevis*	Deformities; delayed growth	Schuytema and Nebeker 1998
	Rana aurora	Limb malformations	Schuytema and Nebeker 1998
Paraquat	*Xenopus laevis* embryo	Tail folding; abnormal gut; oedema	Visnara *et al*. 2000
Fungicides			
Chloranil; dichlone; nabam	*Xenopus laevis* embryo	Disrupted cephalic development; reduced size	Anderson and Prahlad 1976
Triphenyltin	*Rana lessonae* tadpole; *Rana esculenta* tadpole	Slower growth; delayed metamorphosis	Fioramonti *et al*. 1997
Heavy metals			
Lead	*Bufo arenarum* embryo and tadpole	Delayed development; body bending; microcephaly; defective gills; stunted tail	Pérez-Coll *et al.* 1988

Table 1 (*Continued*)

Compound	Species and stage	Effect	Reference
Cadmium	*Bufo arenarum* embryo	Skeletal malformations; delayed development; size reduction	Pérez-Coll *et al.* 1985
	Xenopus laevis embryo and tadpole	Body bending and shortening; microcephaly; eye and tail malformation; reduced pigmentation	Herkovits *et al*. 1997
Arsenic; barium; cadmium; chromium; selenium	*Rana catesbaiana* tadpole	Oral deformities; decreased growth	Rowe *et al*. 1996
Lead; zinc	*Rana luteiventris* tadpole	Reduced weight; reduced motility and fright response	Lefcort *et al*. 1998
Others			
Histamine; imidazole acetic acid	*Bufo arenarum* embryo and tadpole	Delayed development; oedema; organ displacement; abnormal intestine; defective gills; tail bending; heart beat reduction; reduced reaction; swimming in circles	Scolnik *et al*. 1987
Ultraviolet radiation	*Bufo arboreas*	Lordosis; abnormal cornea; hyperplasia	Worrest and Kimeldorf 1976
	Hyla regilla; Rana cascadae	Lordosis	Hays *et al*. 1996

Table 2. Comparative summary of developmental alterations caused by classes of xenobiotics in anuran embryos and larvae.

Target	Effect	Type of compound
Developmental rate	Acceleration	OCs
	Delay	PCBs, OPs, carbamates, pyrethroids, herbicides, fungicides, heavy metals
Weight	Reduction	OCs, OPs, carbamates, pyrethroids, herbicides, heavy metals
	Increase	PCBs
Size	Reduction	OCs, OPs, carbamates, fungicides, heavy metals
	Increase	Carbamates
Body shape	Twisting	OCs, PCBs, OPs, carbamates, pyrethroids, heavy metals
	Dropsy or oedema	OCs, PCBs, OPs, herbicides
Subcutaneous circulation	Haemorrhage	OPs
Tegument	Depigmentation	OCs, PCBs, OPs, carbamates, heavy metals
Notocord	Bending	OCs, PCBs, OPs, carbamates, heavy metals
Gills	Stunted	OCs, PCBs, OPs, carbamates, heavy metals
Snout	Deformities	OCs, heavy metals
Caudal fin	Folding/bending	OCs, PCBs, OPs, carbamates, herbicides, heavy metals
Gut	Protrusion	PCBs, OPs, carbamates, herbicides
Behaviour and locomotion (nervous system)	Hyperexcitability	OCs, pyrethroids, herbicides
	Spasm	Pyrethroids, herbicides
	Paralysis	OCs, OPs, pyrethroids
	Weakness/lethargy	OPs, carbamates, pyrethroids, herbicides, heavy metals
	Altered equilibrium	OCs, OPs
	Circular swimming	OCs, OPs, carbamates

and herbicides (Mulla *et al*. 1963, Cooke 1973, 1977, 1981). Genotoxic alterations in amphibian development have been described in a few studies with pyrethroids (Rudek and Rozek 1992) and herbicides (Clements *et al*. 1997), although many other organic compounds such as polycyclic aromatic hydrocarbons (PAHs) also cause genotoxicity (Sparling 2000).

We may conclude that developmental growth alterations are relatively easy to record as macroscopic biomarkers of effect. However, they are not specific for pesticide class, as are also caused by other organic chemicals and heavy metals.

Signalling, transduction and genetic responses as molecular toxicology biomarkers

The development in molecular genomics and proteomics linked to the recognition of molecular targets provides the basis for more powerful biomonitoring techniques to protect the environment (Adams *et al*. 2001). The subacute and long-term effects of pesticide exposures leading to fertilization impairment, abnormal development and sexual dysfunction in adults involve molecular effectors in the signalling, transduction and genetic response to the stress. Receptors such as the aryl hydrocarbon receptor (Rowlands and Gustafsson 1997), the protein kinase/phosphatase cascades (Matsumura *et al*. 1984) and nuclear transcription factors (Ashida and Matsumura 1998) play essential roles in the toxicity of pesticides and organic pollutants.

A central factor in initiating egg activation at fertilization is a rise in free Ca^{2+} in the egg cytosol, which occurs as a result of its inositol triphosphate-mediated release from the endoplasmic reticulum. Muscarinic receptors coupled to phospholipase C are thought to mediate second messenger release by phosphoinositide breakdown. In amphibians, lipophilic compounds coming in contact with laid oocytes easily pass though the tiny jelly coat and are then able to diffuse into plasma membranes. Membrane-associated processes such as second messenger signalling during fertilization (Kusano *et al*. 1977, Miyazaki 1988) might be then disrupted by lipophilic toxicants. Some compounds intercalate in the bilayer of oocyte membranes, causing phosphoinositide breakdown (Bernard *et al*. 1988) (Table 3). We have studied some of these processes, and found an increased turnover of phospholipids in *Bufo arenarum* oocytes as a consequence of exposure to dieldrin and azinphos methyl. Associated with these changes, blockade of an agonist-gated response via phospholipase C was observed. In addition, there was a retailoring of phospholipids through phospholipase A2 deacylation and lysophospholipid reacylation (Fonovich de Schroeder and Pechen de D'Angelo 1995a, 2000, Caballero de Castro *et al*. 1997). *In vitro* studies showed that dieldrin in fact activates phospholipase C, leading to a desensitized state of the enzyme (Fonovich de Schroeder and Pechen de D'Angelo 1995b). In addition, the free fatty acid pool was increased in dieldrin exposure, mainly as oleic acid (Fonovich de Schroeder and Pechen de D'Angelo 2000), which is the major acyl component in phosphatidyl choline and phosphatidyl ethanolamine from *Bufo arenarum* oocytes. As a consequence of oocyte exposures to OCs, OPs and carbamates, significant decreases in fertilization success have been observed in *Bufo arenarum* (Table 3).

Preliminary studies on molecular biomarkers in *Bufo arenarum* during early development indicate that exposure to OPs affects protein phosphorylation. Related to these changes, activator protein-1 response element (AP-1RE) binding transcription factors are downregulated, while cAMP response element (CRE)-related transcription factors show a biphasic response as determined by electrophoretic gel mobility shift assay (EMSA) (Venturino *et al.* 2001c). c-fos repression and AP-1RE binding downregulation by pyrethroid exposure has been recently reported (Imanura *et al*. 2000). In turn, c-Jun is regulated by c-Jun amino terminal kinase (JNK), which is downregulated by glutathione-*S*-transferase (GST) heterodimerization (Finkel and Holbrook 2000). cAMP-dependent protein kinases and two nuclear transcription factors binding CRE are affected by dioxins (Matsumura *et al.* 1984, Ashida and Matsumura 1998). These results are encouraging in the search for early molecular biomarkers of response to pesticides in amphibians.

Endocrine disrupting chemicals (EDCs) and endocrine active chemicals interfere with signalling systems through hormone receptor binding, altering endocrine and sex determination processes. PCBs and PAHs cause sex reversal in male reptiles, but their effects in amphibians are largely unknown (Sparling 2000). Recent reports have described feminization of male frogs exposed to the herbicide atrazine in the laboratory and in the wild (Hayes *et al*. 2002a,b). Several key developmental and physiological processes are steroid- and thyroid hormone-dependent throughout the amphibian life cycle. Thyroid hormones are essential for metamorphosis and are corticoid-modulated. Both testosterone and oestradiol

Table 3. Compounds affecting receptor binding, second messenger signalling and/or genetic responses as molecular biomarkers in anurans.

Species and stage	Compound	Effect	Reference
Bufo arenarum oocyte	Dieldrin	Increased phosphoinositide turnover; blockade of phospholipase C response	Fonovich de Schroeder and Pechen de D'Angelo 1995a
		Reduced fertilisation rate; activation/desensitization of phospholipase C; phospholipase A2 activation; lysophospholipid acyltransferase activation	Fonovich de Schroeder and Pechen de D'Angelo 1995b, 2000
	Azinphos methyl	Increased phosphoinositide turnover; blockade of phospholipase C response; reduced fertilization rate	Caballero de Castro *et al*. 1997
Bufo arenarum embryo	Malathion; azinphos methyl	Alterations in protein phosphorylation; downregulation of AP-1RE and CRE binding transcription factors	Venturino *et al*. 2001c
Kassina senegalensis larva	DDT	Developmental abnormalities	Hayes 2000
Rana pipiens adult	Atrazine	Aromatase induction; decrease in testosterone levels and hermaphroditism in males	Hayes *et al*. 2002b
Xenopus laevis oocyte	Maitoxin	Phosphoinositide breakdown	Bernard *et al*. 1988
Xenopus laevis adult	DDT; toxaphene; dieldrin	Vitellogenin induction	Palmer *et al*. 1998
	Tetrachlorbiphenyl; DDT	Oestrogen receptor binding	Lutz and Kloas 1999
	Atrazine	Aromatase induction; decrease in testosterone levels and hermaphroditism in males	Hayes *et al*. 2002a

inhibit amphibian metamorphosis (Hayes 2000). Few effects of EDCs have been reported in amphibians to date. Dioxins affect metamorphosis by targeting the thyroid system. Developmental abnormalities were observed in *Kassina senegalensis* exposed to dichlorodiphenyltrichloroethane (DDT) (Hayes 2000) (Table 3). In adult *Xenopus laevis* females, thyroid hormones stimulate oestrogen receptors, enhancing oestrogen-mediated induction of vitellogenin (May and Knowland 1980). Vitellogenin has become a recognized biomarker of effect for EDCs. OCs such as DDT, toxaphene and dieldrin may act as pro-oestrogens, inducing vitellogenin in male adults (Palmer *et al*. 1998). The level of binding to the liver oestrogen receptor has been effectively assessed for several aromatic compounds, including PCBs and DDT, in *Xenopus laevis* adults (Lutz and Kloas 1999). Another proposed mechanism of feminization in male *Xenopus laevis* and *Rana pipiens* adults exposed to atrazine is aromatase induction and testosterone conversion to oestrogen (Hayes *et al*. 2002a,b).

Acetylcholinesterase and other esterases

Acetylcholinesterase (AChE) activity is crucial at cholinergic synapses and muscular plates, contributing to the cessation of acetylcholine stimulation at the postsynaptic membrane once the nervous signal has been transmitted. AChE is considered the main target of OP insecticides, which bind irreversibly to the enzyme active site as suicide inactivators, and the reduction in its activity is generally associated with lethality. Nevertheless, AChE and butyrylcholinesterase (BChE) have another relevant role in the development of the brain and nervous system during early embryogenesis (Drews 1975, Krejci *et al*. 1991). AChE is recognized as a specific biomarker of effect in pesticide exposures (Adams 2001). Both BChE and the detoxifying activity of carboxylesterases (CEs) have been proposed as biomarkers of effect for OP and carbamate pesticides (Bartowiak and Wilson 1995, Sánchez *et al*. 1997). However, the use of AChE as a biomarker in carbamate and OP exposure is less well quantified in amphibians than in other vertebrate classes (Henry 2000).

We have evaluated the developmental pattern and seasonal variations in the activities of AChE, BChE and CEs *in Bufo arenarum* (Caballero de Castro *et al*. 1991, Caballero de Castro 2000). AChE and BChE activities were detectable from gastrulation on, and peaked at 5 days of development (complete operculum stage) in summer embryos. CE activity was detected in oocytes and the earliest stages of embryonic development, and also peaked at day 5 (Figure 1). Winter clutches showed delayed patterns of these enzymes, associated with a slower embryonic development.

OPs suppress the activity of these three enzymes or delay their appearance and progression in embryos recovered from transient exposures (Rosenbaum *et al*. 1988, Caballero de Castro *et al*. 1991). Embryos treated with malathion for 5 days recover between 25 and 40% of the peak activity after a delay of 2–3 days. Embryos treated for 3 days recover between 40 and 65% of the peak activity after a delay of 1 day. In *Bufo arenarum* larvae, the recovery times from exposure to different OPs (malathion, parathion and azinphos methyl) ranged from 1 to 7 days to achieve 70–100% AChE activity (Venturino *et al*. 1992, 1993, 2001b, Anguiano *et al*. 1994).

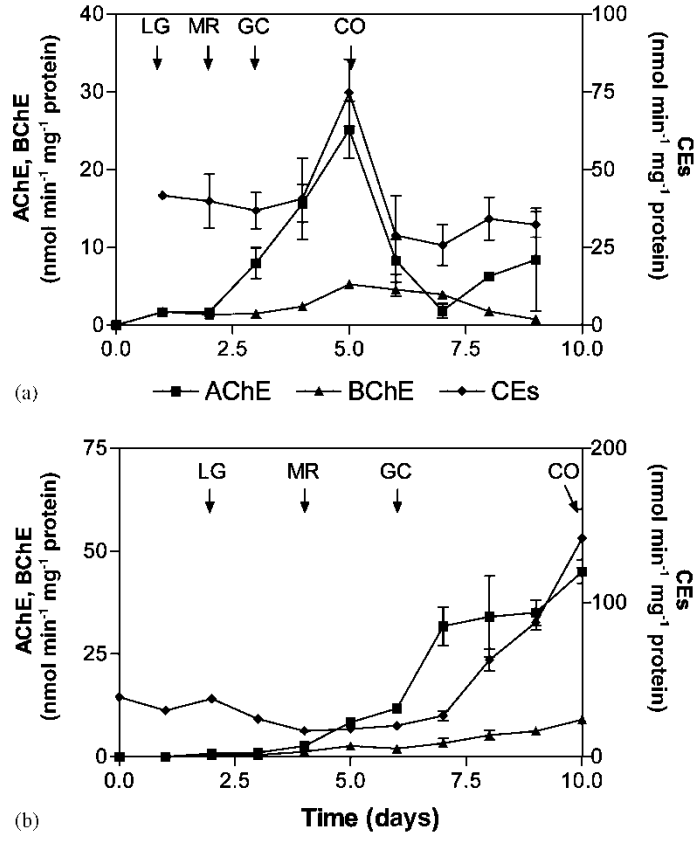

Figure 1. Developmental pattern and seasonal changes in esterase activities during *Bufo arenarum* embryogenesis. Esterase activities were determined in embryos obtained by *in vitro* fertilization at different stages of development during the summer (a) or winter season (b). The esterases analysed were acetylcholinesterase (AChE), butyrylcholinesterase (BChE) and carboxylesterases (CEs). Stages achieved at days of development are indicated by arrows: LG, late gastrulae; MR, muscular response; GC, gill circulation; CO, complete operculum. Data from Caballero de Castro 2000.

Significant inhibition and a fast recovery were found with carbamate carbaryl. The OP temephos inhibited BChE in *Rana clamitans* larvae, but increased AChE activity (Sparling *et al*. 1996). This effect was attributed to inefficient oxidation of the OP to the oxon form, the active inactivator of AChE. Other organic compounds such as OCs may also affect cholinesterases; for instance, dieldrin decreases both AChE and BChE activities in *Bufo arenarum* embryos (De Llamas *et al*. 1985).

Determination of the effects of exposure to OPs or carbamate, as well as those of other toxicants, needs to take into account the species-specific variations associated with development and season. In some species such as *Xenopus laevis* tadpoles, AChE is highly resilient to anticholinesterase agents (Shapira *et al*. 1998). The important fluctuations in AChE, BChE and CEs due to seasonal and developmental changes observed in *Bufo arenarum* embryos make the assessment of control reference values difficult. The relatively fast recovery of the activities after an episodic exposure to pesticides may also complicate the assessment of field

effects. Consequently, the use of these enzymes as biomarkers in anuran species requires particular caution.

Biochemical responses related to pesticide oxidative metabolism and detoxification

Pesticide metabolism is generally linked to oxidative stress directly by generation of reactive oxygen species (ROS) through enzymatic transformation or by consumption of reduced co-substrates such as reduced nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH) (Finkel and Holbrook 2000). Mixed-function oxidases (MFOs) are involved in the oxidative metabolism of practically all pesticides and contribute to ROS generation. Amphibians appear to have a less developed P450-dependent MFO system than that found in mammals (Sparling 2000). P450 induction is low in amphibians compared with other classes, reducing its effectiveness as a biomarker of effect (Eartl and Winston 1998, Huang et al. 1998). Moreover, a very large natural variation in the activity of several MFO components has been found in *Rana temporaria* adults, probably resulting from the hormonal changes associated with spawning (Harri 1980). MFO induction was demonstrated in malathion-exposed *Bufo arenarum* larvae (Venturino *et al*. 2001b) (Table 4).

Reduced glutathione (GSH) is perhaps the most important ROS scavenger participating in the control of the intracellular redox status (Finkel and Holbrook 2000). Some pesticides are detoxified by GSH conjugation or demethylation through a pathway involving GST activity, and alterations in the cellular antioxidant defence have been associated with OP exposures (Hai *et al*. 1997). Antioxidant enzymes, glutathione and lipid peroxidation levels are relevant biomarkers in aquatic toxicology (Doyotte *et al*. 1997). We have evaluated these different pathways related to pesticide oxidative metabolism in exposed *Bufo arenarum* embryos and larvae. Different OC and OP insecticides affect the reduced GSH pool, measured as acid-soluble thiols (ASTs), in toad embryos and larvae (Anguiano *et al*. 2001, Venturino *et al*. 2001a). Malathion, azinphos methyl and lindane decrease the reduced AST level to nearly 50% in embryos, whereas parathion and dieldrin do not affect it (Table 4). Larval stages are mainly affected by malathion and azinphos methyl, which deplete AST by up to a 30% of control levels. Nevertheless, all the pesticides tested induce GST activity in *Bufo arenarum* larvae, suggesting the involvement of this enzyme in their metabolism. Malathion also decreases total (reduced plus oxidized) AST, and methyl GSH has been detected in exposed toad larvae, corroborating the participation of the GST pathway in the detoxification of this insecticide (Venturino *et al*. 2001a,b). Thus, these biochemical parameters are biomarkers of the oxidative metabolism of some insecticides in exposed *Bufo arenarum* embryos and larvae. It is worth noting that mild exposures to these insecticides do not lead to oxidative stress, as lipid peroxidation levels remain similar to those in control tadpoles. GSH and metallothioneins, commonly used as biomarkers in metal exposure, have also been evaluated in exposed amphibians (Suzuki and Akitomi 1983, Suzuki and Kawamura 1984, Vogiatzis and Loumbourdis 1998). Other metabolic alterations have been studied in the ovary of adult *Bufo arenarum* females in the search for new biomarkers for metal exposure. The enzyme glucose 6-phosphate dehydrogenase is

Table 4. Biomarkers of oxidative stress, antioxidant defence and enzyme detoxification in anurans.

Biomarker	Species and stage	Compound	Effect	Reference
MFO	*Bufo arenarum* larva	Malathion	Induction	Venturino *et al*. 2001b
	Rana pipiens	Pentachlorobiphenyl	Induction	Huang *et al.* 1998
	Rana temporaria adult	DDT; seasonal variations	No effect	Harri 1980
GST	*Bufo arenarum* embryo and larva	Malathion; parathion; azinphos methyl; lindane; dieldrin	Induction	Anguiano *et al*. 2001; Venturino *et al*. 2001c
Metallothionein	*Rana ridibunda* adult	Cadmium	Induction	Vogiatzis and Loumbourdis 1998
	Rana catesbeiana larva and adult	Cadmium	Induction	Suzuki and Akitomi 1983
	Bombina orientalis adult; *Bufo bufo japonicus* adult; *Hyla arborea japonica* adult	Copper; zinc	Induction	Suzuki and Kawamura 1984
Glucose 6-phosphate dehydrogenase	*Bufo arenarum* adult	Zinc	Inhibition	Fonovich de Schroeder *et al*. 2000; Naab *et al.* 2001
GSH	*Bufo arenarum* embryo	Malathion; azinphos methyl; lindane	Decrease	Anguiano *et al*. 2001
	Bufo arenarum larva	Malathion	Decrease	Venturino *et al*. 2001a
	Bufo arenarum adult	Zinc	Increase	Fonovich de Schroeder *et al*. 2000; Naab *et al.* 2001
	Rana ridibunda	Cadmium	Increase	Vogiatzis and Loumbourdis 1998
GSH+GSSG (total)	*Bufo arenarum* larva	Malathion	Decrease	Venturino *et al*. 2001a
Lipid peroxidation	*Bufo arenarum* larva	Malathion; parathion; dieldrin; lindane	No effect	Venturino *et al*. 2001a,c
Polyamines	*Bufo arenarum* embryo	Malathion	Decrease	Venturino *et al*. 2001c
		Malathion (sublethal)	Increase	Venturino *et al*. 2001a
	Bufo arenarum larva	Malathion	No effect	Venturino *et al*. 2001a

GSH, reduced glutathione; GSSG, oxidized glutathione.

able to bind zinc, which is considered a micronutrient that does not normally accumulate in the tissues. This enzyme is inhibited by long-term exposure of females to zinc in Ringer solution. As the result of this inhibition, the oocytes are subjected to oxidative stress and respond with an increase in GSH content (Fonovich de Schroeder *et al*. 2000, Naab *et al*. 2001) (Table 4).

The levels of polyamines, which are essential for a wide range of biological processes, are altered in severe cellular stress and toxicosis associated with apoptosis via hydrogen peroxide generation and GSH depletion during embryonic development (Coffino and Poznanski 1991). We examined polyamine levels as biomarkers of the effects of pesticide on the embryogenesis of *Bufo arenarum*. Malathion concentrations causing acute toxicity decreased polyamine levels in middle and late embryonic stages (Venturino *et al*. 2001c), and this effect was related to impaired development and abnormal morphogenesis (Table 4). Sublethal exposures to malathion caused an increase in putrescine concentration at the end of embryonic development as a recovery response, while no effects were observed in larvae (Venturino *et al*. 2001a). Thus, polyamines may be useful biomarkers of pesticide effect and recovery responses during amphibian development, depending on the stage and level of exposure.

Conclusions

Different biochemical, physiological and morphological parameters may be needed at different stages in anuran development in order to assess exposure and response to contaminants.

Reductions in fertilization are partially associated with changes in the oocyte membrane phospholipid turnover, and alterations in the muscarinic acetylcholine receptor signalling pathways and other membrane-associated enzyme activities.

During early embryonic development, different chemicals may affect the nuclear transcription factors regulating gene expression, thus altering a cascade of responses. The identification of transcription factors associated with the exposure of amphibians to xenobiotics is highly promising. However, these methods are likely to complement other biomarkers. Among them, GSTs are induced by pesticides, in some instances lowering reduced AST pools (generally associated with GSH) and total GSH.

After gastrulation, malformations are commonly found following chemical exposure. However, developmental alterations in anurans are not specific to the chemicals involved and may be difficult to assess in the field.

Esterases show seasonal and developmental patterns that are abolished or delayed by insecticide exposures. Esterase activities show relatively fast recoveries after brief exposures, and their sensitivity to anticholinesterase agents vary among species, reducing their usefulness as biomarkers.

GSH, GSTs and metallothioneins respond to oxidative and metal stress in exposed embryos and tadpoles. MFO activities in tadpoles are low and are poorly induced by xenobiotics. Polyamines are depleted at the end of embryonic development by lethal exposures, and are related to teratogenesis, reduced growth

and reduced survival. On the other hand, sublethal exposures cause stress-related putrescine increases, associated with MFO induction.

Finally, in adult anurans a few specific biomarkers such as vitellogenin for EDCs and metallothioneins and glucose 6-phosphate dehydrogenase inhibition for heavy metals have been described.

In conclusion, the use of several biomarkers with different endpoints is needed to link exposure to response, and to provide better predictive tools for the environmental protection of endangered anuran species. These biomarkers, among others coming into use, provide a range and diversity of biological responses in toads and frogs that may be useful in environmental risk assessment after being validated in the field (Adams *et al*. 2001). Applying a variety of biomarkers in predictive ecotoxicology will improve the interpretation of effects and will help to ensure their significance in impact assessment.

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