Methods for Assessing the Effects on Chemicals on Reproductive Functions
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Assessment of the Effects of Chemicals on the Reproductive Functions of Reptiles and Amphibians

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1 INTRODUCTION

Amphibians and reptiles are regarded as rather primitive vertebrates. They are poikilothermic, in which they differ from birds and mammals; on the other hand, reptiles, like birds, produce eggs, while amphibians mostly produce spawn, like fish.

The risk in not maintaining a uniform body temperature lies in vulnerability to extreme temperatures. There are advantages: food intake is proportional to the actual body temperature; this is not only economic, but it is a survival mechanism when the environment is hostile.

Reptiles and amphibians are most abundant in the tropics but are, as well, spread far in the temperate zones. They are very prolific with a high degree of environmental adaptation. The economic benefit of these groups is limited except for crocodiles, alligators, green turtles and frogs.

Reproduction differs in amphibia and reptiles. Most reptiles produce eggs; but there are differences, e.g. tortoises and geckoes have hard-shelled eggs, laid in dry places, while in other species the eggs may have soft, flexible shells laid in moist sand or earth or dead vegetation. In a few species the eggs are retained within the body of the mother. In all cases the newly hatched young are minor copies of the parents, and all are independent. Amphibians produce eggs, with a gelatinous coating, usually laid in water. They hatch into tadpoles, which eat and grow before metamorphosing into young, parent-like animals. Only a few genera give birth to larvae or fully formed infants, or lay eggs which hatch into metamorphosed young. The larvae, or tadpoles, normally live in water.

This discussion of reproductive injury is primarily concerned with the effects of chemicals on the formation of eggs and sperm, embryogenesis, larval development, and growth and metamorphosis.

There is little information available on the toxic effects of environmental contamination on amphibians and reptiles compared, for example, to birds.

Specific information on the effects of external factors on reproduction is even more poorly documented.

In addition to the threat of pesticides to these groups, injury can occur from heavy metals, such as copper and cadmium, oil spills in water, as well as a low pH of water. Genetic damage may occur from a number of chemicals. Environmental contamination seems to affect amphibians and reptiles to about the same degree as other animals.

Physiological differences will, however, contribute to differences in response. For example, the water-vapour conductance in eggs is highly variable in turtles and reptiles. A snapping turtle egg (*Chelydra serpentia*) has a conductance to water 55 times higher than the avian egg, whereas the soft-shell turtle egg (*Trionyx spiniferus*) has a conductance only 5–6 times higher than the avian egg and that of the American alligator (*Alligator mississippiensis*). This implies possible differences in the intake of water-soluble agents by the egg, in this case the difference is about 10 times (Packard *et al.*, 1979). It has been shown that insecticides (DDT and dieldrin) do enter the eggs of the cottonmouth snake (*Agkistrodon mokeson*) (Fleet, *et al.*, 1972).

In anurans, the skin is the important organ for water uptake, lessening the need for actual drinking. Thus, a water-soluble agent may be absorbed into the body from the aqueous environment whereas a water-living snake can drink from uncontaminated water.

2 EFFECTS ON OOGENESIS AND SPERMATOGENESIS

In the African clawed toad (*Xenopus laevis*), the early cleavage stages of the eggs are sensitive to a halogenated analogue of the normal thymidine, incorporated during DNA synthesis. This pyrimidine, 5-bromodeoxyuridine, will be mistaken for thymidine during mitosis and block development at the blastula stage, leading to point mutations in the newly formed DNA strands. The effect is greater with echinoderm eggs than amphibian eggs, which may be explained by the fact that more thymidine, proportionally, is present in amphibian eggs (Sala and Conte, 1975).

Early chemical injury of oocytes can occur in the RNA. Oocytes from *Xenopus laevis* treated with the aminopiperazin radical will cease developing in a pachythene meiosis phase, ultimately resulting in the non-production of eggs (Steens, 1977).

A similarity in reaction between mammals and amphibians and reptiles was demonstrated in the lizard and the toad. Quinacrine, which interferes with sperm production in mammals, was tested for its effect on steroidogenesis in lizards and histological reactions in the toad. With the toad, the drug produced testicular lesions and changes in cell morphology. In the lizard (*Psammophilus dorsalis*), the compound caused weight loss of the testes, epididymis, vas deferens and kidneys and a total cessation of spermatogenesis. It seems to interfere with steroid biosynthesis and metabolism, indicated by the presence of accumulated amounts

of the precursor of steroids, sudanophilic lipids (Shivakuma and Devaraj Sarkar, 1979).

It is well known that cadmium is extremely toxic to mammalian testes and to spermatogenesis. It has been questioned whether animals with abdominal testes react in the same way as animals with scrotal testes do. Frogs and toads were therefore exposed to cadmium by means of intraperitoneal or subcutaneous injections, followed by incubation under laboratory conditions. Sexually active frogs (*Rana tigrina*) showed a mild shrinkage of the testes after 48 hours which later regenerated, with relatively prompt production of spermatids and spermatozoa. On the other hand, with sexually inactive frogs of the same species, formation of spermatozoa was not observed until a week after injection. The effect of cadmium may bring about a release of gonadotropins which in turn initiated spermatogenesis (Matur and Ramaswami, 1976).

In toads (*Bufo melanostictus*) the injection of cadmium chloride caused a significant decrease in numbers of secondary spermatogonia and primary spermatocytes after 7 days. The suggestion of the authors is that, in the toad, cadmium suppresses spermatogenesis possibly by increasing testicular steroid hormone synthesis (Biswas *et al.*, 1976). Cadmium seems to interfere at the level of hormone regulation, although the mechanism is not well understood.

In 1926, Dilling and Healy stated that ions of lead, copper, zinc, thorium, beryllium and thallium were all suspected of disturbing the germination of the frog spawn and the growth of tadpoles. The methods were rather crude, but the conclusion was that lead was by far the most active antifertility agent.

3 EFFECTS ON EMBRYOGENESIS

The response to the herbicide preparation, Weedex, caused a total halt to reproduction in a frog pond. Weedex had been sprayed on a railway track nearby, and the spawn found in the pond were cloudy and did not hatch. Captured *Rana* frogs from the pond laid spawn in a tank, and the hatching result was poor from these also. The surviving tadpoles were three times as heavy as normal, and all died before metamorphosis. The frogs could not reproduce again until the pond was cleaned and the bottom dredged. No information was given as to spraying intensity or concentration in water. It was clear that the substance remained active in the sediment after the pond had been kept dry for a long period (Hazelwood, 1970).

Well-developed spawn of the common frog, *Rana temporaria*, was not penetrated by DDT at 0.5 p.p.m. for 24 hours. The solution was prepared by adding DDT in ethanol to Holtfreter's amphibian saline; the controls were kept in the same medium without DDT. However, when freshly laid spawn, which swells by taking in water, was treated with DDT, the hatched tadpoles showed typical hyperactive behaviour 8–13 days after hatching, and development was retarded (Cooke, 1972).

Extremes in temperature and pH will cause death of the embryos of the

salamander, *Ambystoma maculatum* and *A. jeffersonianum*. Mortality occurred before gastrulation, gill formation, or hatching. The rate of development was not influenced by pH (Pough and Wilson, 1974).

Temporary ponds were the site of a field study by the same author. Embryonic mortality of spotted salamander (*Ambystoma maculatum*) was found to be correlated with a low pH caused by acid precipitation. Environmental differences, such as different soil, proximity to road, etc., were noted but not controlled (Pough, 1976).

A treatment of *Rana pipiens* eggs with copper sulphate at concentrations of 0.04–1.56 mg Cu/l did not cause death or damage; the eggs were stripped from a female and soaked in sperm suspension and then treated (Landé and Guttman, 1973).

4 EFFECTS ON LARVAL DEVELOPMENT

Copper, as noted, has little or no effect on amphibian eggs. When hatched, however, tadpoles are very sensitive to inorganic copper; growth is grossly reduced and development retarded to the point of no metamorphosis. The LD_{50} (72 hours) was estimated at 0.15 mg Cu/l (Kaplan and Yoh, 1961; Landé and Guttman, 1973).

The cytotoxic effect of copper in amphibians and reptiles does not seem to have been investigated as thoroughly as, for example, cadmium. Copper is toxic to adult frogs, but at far higher concentrations than cadmium. This may mean that copper action can be partly described as a 'metal-salt reaction', e.g. excessive mucus formation, gill coagulation, eye irritation, etc. The fact that bigger tadpoles, exposed to copper salts, have a higher survival rate than smaller individuals could be a result of a higher degree of damage to the outer gills of the small larvae and the proportionally greater surface of the small animal.

With frogs and toads, it has been demonstrated a number of times that DDT and its residues are harmful to tadpoles as well as to the adults. Treated tadpoles become hyperactive, which in turn retards development; the smaller, hyperactive tadpoles are more likely to be victims of predation than the normal tadpole. The weight loss in connection with hyperactivity is probably a result of feeding difficulties in this stage of behavioural disturbance. Also, skeletal deformities occur such as twists in the spine. With higher doses, the hyperactive stage will be followed by loss of equilibrium, lethargy and death. These experiments involved acute doses of DDT, in concentrations ranging from 0.0008 to 10 p.p.m. DDT. The solutions were made up from ethanol–DDT mixtures in amphibian saline.

There were differences in the response at different ages and at different concentrations. Tadpoles with developing hind limb-buds of both *Rana pipiens* and *Bufo bufo* were more vulnerable than others. At concentrations around 0.01–0.02 p.p.m. DDT, the typical frantic behaviour occurred as well as twisted spine. No actual LD_{50} values are calculated for the tests, but high concentrations

gave rise to uncoordinated behaviour soon after the tadpoles were placed in them and mortality in the groups at 1 p.p.m. and 10 p.p.m. DDT was high.

It appears that the hyperactive stage occurs at tissue concentrations around 2–3 p.p.m. in frogs and at 3–4 p.p.m. in toads. At the tail resorption and metamorphosis stage, frog tadpoles are more susceptible to DDT residues than toads. Chronically treated tadpoles (0.0001–0.001 p.p.m. *p,p'*-DDT) showed more rapid development and growth, and no behavioural disturbances even at tissue concentrations of 2–5 p.p.m. The twisted spine was fairly common, however, among treated tadpoles. At metamorphosis these residues caused mortality among the young frog imagos, as the lipid reserves were exhausted and the DDT released. Young toads treated in the same way were not affected after metamorphosis (Cooke, 1970, 1972, 1973).

It has been suggested that insecticides lower the resistance of the tadpole to fungi, possibly implying that the agent may be only indirectly responsible for the observed deformities (Cooke, 1975a).

DDT exhibits its action through its metabolites as well, e.g. DDCN. This compound was found in the sediment of a Swedish lake and presumed to be widespread in biologically active mud and sediment. Tadpoles of the common frog were exposed to DDCN under laboratory conditions. Residues were found in the tadpoles from treated groups exposed to ≥ 0.1 p.p.m. *p,p'*-DDCN but not from lower concentrations. The residues were not detected when tadpoles were moved into uncontaminated water. At the highest concentration, 1 p.p.m., the tadpoles acted abnormally, were lethargic and very weak. Malformation of the tail occurred, a similarity with DDT-treated toad tadpoles. The deformity and the behaviour were reversible in clean water. Low levels of DDCN also seem to accelerate the development of the tadpole, as with DDT. On the whole, this compound is less toxic to frog tadpoles than DDT (Cooke, 1975b).

On exposing anuran tadpoles to DDT, a significant increase of pituitary melanocyte-stimulating hormone, MSH, was found; a direct effect of DDT upon the hypothalamus was postulated. The effect of MSH in excess is wakefulness, which can be measured from EEG data. The exposure in this case was made on *Rana clamitans* larvae in the limb-bud stage. DDT was added directly to the aquaria as a wettable powder. One group of tadpoles was exposed to 0.1–0.5 p.p.m., the other 0.5–0.8 p.p.m. DDT. Nothing is mentioned about media or temperature (Peaslee, 1970).

In a toxicity test of 19 pesticides, insecticides were, generally, 10 times more toxic than herbicides when tested on tadpoles of the frog. (*Pseudacois triseriata*). With tadpoles of the toad (*Bufo woodhousii*) the pattern was approximately the same. Some of the differences in response might be explained from differences in the ages of frogs (1 week) and toads (5 weeks) since it is known, for example, that DDT sensitivity differs with age in tadpoles. The signs of pesticide poisoning followed a predictable pattern: irritability, loss of equilibrium and death. Methodologically this experiment was thorough. It was carried out as a

static bioassay, beginning with a pilot test for selecting concentration intervals. The test animals were hatched in the laboratory. The stock solutions were made in ethanol, and the tests were carried out in 5 litre aquaria (Sanders, 1970).

As with mosquitos, it appears that DDT-exposed anurans may develop resistance as, for example, in the adult cricket frogs, *Acris crepitans* and *A. gryllus*, living in the cottonfields of southern USA (Boyd *et al.*, 1963).

Resistance tests showed a possible cross-resistance between aldrin and DDT which may result from the fact that both are chlorinated hydrocarbons (Vinson *et al.*, 1963).

Tolerances to aldrin, dieldrin, endrin, toxaphene and DDT were tested on two frog species (*Acris crepitans, Acris gryllus*) and one toad (*Bufo woodhousii*). The tadpoles were collected from agricultural (pesticide-exposed) areas and from non-agricultural land in order to seek possible resistance to the agent. The relative toxicities of the five insecticides tested were consistent for all three species tested. Endrin was most toxic, followed by aldrin and dieldrin, which were about the same. DDT and toxaphene were least toxic and produced similar levels of mortality. No resistance could be detected; that is, differences in susceptibility relatable to whether the animals came from pesticide-treated as compared to untreated soils were not found. The method used was a 36-hour TL_{50}; temperature range was 80 ± 2°F and the animals were of equal size (18–30 mm) (Ferguson and Gilbert, 1967).

Another pesticide Cyanatryn, chemically related to Weedex, was tested. When used at recommended levels (0.4 p.p.m.), tadpoles of *Rana temporaria* stopped feeding, acted lethargic and behaved in such a way that they were likely to become victims to the newt or other predators. The risk of magnification of concentrations or effects is obvious (Scorgie and Cooke, 1979).

5 METHODS DEVELOPMENT

With amphibians and reptiles there is an urgent need for the development of relevant tests and the identification of suitable test organisms. Many of the techniques used for fish might apply to amphibian testing; similarly, tests used with birds may, with appropriate alteration, might be useful reptiles.

It is not possible to consider reptiles and amphibians as a single group in respect to the effects of chemicals. For example, food sources differ; amphibians are often at the low end of the food chain, while many snakes can be carnivores at the upper end. Also, the yolk of the snake egg is proportionately larger than the yolk-sac of the amphibia, which means a higher proportion of the snake mother's fat will be mobilized into the egg, and with the fat may come fat-soluble substances such as pesticides. Amphibia, on the other hand, will be exposed to water during embryogenesis and the early stages after hatching. Pools and ponds are often collection and sedimentation basins for water-carried materials, such as chemicals. This may give a higher chemical exposure to animals in water than to the animals on shore.

Also, the volume/surface ratio must be considered in respect to exposure to harmful substances. A small embryo has a proportionally greater surface than a large one, and the response is likely to be more marked. In the choice between two test species, the one with the proportionally smaller embryos may have an advantage in sensitivity.

Three general approaches are available to assess effects on reproduction:

(1) acute toxicity;
(2) effects other than death, including early warning signs; and
(3) effects from chronic exposures.

With amphibians and reptiles, the tests fall mostly into group (1) or (3). The sublethal testing is by far the most interesting at the population level and, as well, for ecological understanding. The importance of early warning systems for non-target organisms is vital; animals living in water part or all of their life are not easily observed, and behavioural alteration is unlikely to be discovered. The earliest warning today is likely to come from nature enthusiasts and sportsmen which, when accurate, can only serve to confirm that harm is already done.

The selection of test animals and species include such criteria as the holding and breeding of the animals at reasonable cost, susceptibility to toxicants representative for the class or phyla, and suitability for testing, e.g. production of numerous offspring.

OECD (1979) has suggested the use of the African clawed toad for testing embryotoxicity; otherwise testing procedures for reptiles and amphibians having official or semi-official status appear to be non-existent. The hope for the future is to widen the concept of toxicity testing to keep in mind the total environment, including organisms other than the target organism. For evaluating a new product or a chemical compound, single, standardized methods for toxicity should be used, as well as tests for possible synergistic effects. After the level of toxicity is clear, the effect on the whole system would be studied to the extent practicable. The possibilities of accumulation, or long-term damage, or sublethal reproductive injury are, in the long run, far more harmful than highly toxic, acutely active substances, as the former will act in a subtle way and may cause considerable damage before it is even noticed. This is especially a danger with groups or organisms so little observed and valued as amphibians and reptiles.

6 COMMENTS. SUMMARY AND RECOMMENDATIONS

This review shows clearly that the main concern during the last 20 years has been non-target exposure of anurans to pesticides. There are very few reports on heavy metals, and poisoning of amphibians and reptiles by methylmercury is not once discussed. Compared to the mass of research on methylmercury in fish and birds in the early seventies, this can have but one explanation: amphibians and reptiles are not of sufficient interest. Economically only a few species are hunted or

sought, but ecologically they have great importance as bioaccumulators in the food chain.

In the anurans it seems possible to disturb oogenesis and spermatogenesis at the DNA and RNA level. However, whether this is possible without injecting the substance into the animals is not known. Pesticides in the water can damage the eggs in at least two ways: the substance may enter the egg in the swelling phase, or it can destroy the gelatinous coating by coagulation. Copper salts seem not to damage the egg.

Tadpoles from insecticide-treated spawn show, within a week, the same signs as tadpoles treated directly with the same substance. Gill-bearing tadpoles are more sensitive than tadpoles with inner gills; tadpoles developing leg buds are even more susceptible to insecticide treatment. If residues are left in the body, the metamorphosed imago may die from the mobilization of fat containing pesticides. Frog tadpoles are generally more sensitive than toad tadpoles. Newt tadpoles are rarely tested but show the same pattern of reactions, including death. Herbicides may be less toxic than insecticides, but there are exceptions; nothing is known about possible synergism between these pesticides.

Cadmium is reported to block spermatogenesis in toads under certain conditions. Mutagenic effects from metals have been observed in other species, but extrapolation to reptiles and amphibians is uncertain. No teratogenic effects of metals in amphibians and reptiles have been described. Effects in other species have been described, but again extrapolation is uncertain.

Methods reviewed have varied widely, and it is seldom possible to compare data from one study with another. The degree of methodological refinement of toxicity studies on amphibia and reptiles is low compared to that on fish and other species.

There is an urgent need to develop more informative test procedures for examining the effects of chemicals on the reproductive function in reptiles and amphibians. Such development can receive guidance from tests developed for other organisms, especially fish and birds. Although the development of methods is still too primitive for standardization, this objective should be kept in mind.

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