Bull. Inst. Oceanogr. & Fish. ARE, 10 (1984) : 111–121.

POTENTIAL USE OF PHYSIOLOGICAL CHANGES AS INDICES OF STRESS IN THE TELEOST, CYPRINUS CARPIO.

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ABSTRACT

The study concerns the effects of in vivo exposure to sublethal concentrations of the insecticide DDT or the herbicide atrazine (100 and 500 µg/liter) on plasma cortisol levels, gill ATPase activities and brain acetylcholinesterase activity in the carp, **Cyprinus carpio.** Blood and tissue samples were taken after different lengths of time (3, 6, 12 and 24 hr.). Controls were. treated parallel. Plasma cortisol levels showed a strong elevation with both compounds at all concentrations, the reaction on DDT was much stronger. Gill Na^+, K^+-ATPase activity was inhibited after exposure to both compounds. Exposure to DDT did not influence gill Mg^{+2}-ATPase activity, atrazine at both concentrations inhibites the enzime activity after 12 hr. DDT has a more pronounced effect than atrazine on brain acetylcholinesterase (AChE) activity. The practicality of using the changes in the measured parameters as criteria for water quality are given.

INTRODUCTION

The current widespread presence of pesticides in world water ways has elicited much interest in the mechanism of their toxicity. Many workers have attempted to identifiy certain biochemical and physiological parameters as indices of stress in aquatic animals (Dillon and Lynch, 1981; Melancon et al., 1981; Simon et al., 1983). The value of physiological indices of stress lies in the fact that they may be early warning signs, signaling a possible deterimental effect prior to wholesale changes in the population and community structure and function.

Pesticides posses a neurotoxic action and inhibit enzymes. Inhibition of acetylcholinesterase (AChE) from fish has been reported by Goodman et al. (1979) in **Cyprinodon variegatus,** by Koudinya and Ramamurthi (1979) in **Sarotherodon mossambicus,** and by Klaverkamp and Hobden (1980) in **Salmo gairdneri.** Reduced Na^+- K^+- ATPase activity in fish has been reported

in vivo and in vitro following exposure to a wide varity of lipophillic xenobiotics (Leadman et al., 1974; Kuhnert et al., 1976).

Hormones, primarily the corticosteroids, are believed to mediate the stress response by serving as a stimulus between the neural transducer and other factors of the General Adaptation Syndrome which consists of the physiological responses elicited by most stressing agents (Selye, 1976; Schreck and Lorz, 1978; Tomasso and Parker, 1981).

The carp, **Cyprinus carpio,** was introduced into Egypt in 1949 (Eisawy and ElBolock, 1975). The fish represent an acceptable human food and have been reared by many aquaculturists in combination with other species. In Egypt, the average annual consumption of pesticides is relatively high (El-Sabae, 1980). Moreover, many of the commercial fish ponds are located adjacent to cotton field and other agricultural areas. The intensive spraying operations during the growth seasons invariably cause contamination of the ponds.

The present investigation was designed to determine the feasibility of using plasma cortisol levels, brain AChE activity and gill ATPase activity as indicators of stress and pre-mortal sign of pesticides toxicity during short-term exposure of the carp to different sublethal concentrations of the insecticide DDT or the herbicide atrazine.

MATERIAL AND METHODS

Maintenace and preparation of the fish.

Carp weighing 30-50 gm were brought to the laboratory from commerical fish pond, and maintained in 400-liter tanks for at least two weeks. The holding tanks recieved a continuous supply of tap water (temp. 22 $\pm$ 2° C). A photoperiod of 16L:8D was maintained. Dissolved oxygen was never below 90 % saturation. Acidity was mointered regularly (pH 7.3 $\pm$ 0.4). Fish were fed on a carp diet ad lipitum once every other day, feeding was interupted 24 hr before the experiments. A day prior to the application of DDT or atrazine, fish were transfered to a 20 1 test glass aquaria in the proportion of 8 fish per aquarium.

Reagents

DDT and atrazine were obtained as technical grade from Riedel Ltd, and stock solutions were prepared in ethanol. Toxicants were added to the glass aquaria in very small volumes of solvent. Controls contained equivalent volumes of solvent.

Experiments

They were initiated by adding the ethanolic solution of DDT or atrazine to the water to have final concentration of 100 or 500 ug/liter. The fish were sacrifieced in group of 8, unless other wise stated, after exactly 3, 6, 12 and 24 hr. Carps were caught by hand net and quickly anaesthesized

in MS 222 solution. Sacrifieces were made at the same time each day to preclude the potential effects of diurnal fluctuation on enzyme and hormone levels. Blood was obtained by direct puncture of the heart using a heparinized glass pipet, plasma was separated and stored in a deep freeze. After blood sampling the fish were killed by incision, posterior to the brain, and the brain dissected and directly frozen. Individual gill arches were separated and gill filaments removed and frozen.

Enzyme assays

Na^+ - K^+-ATPase

The gill filament of the firest gill arch, was homogenized with 12 complete storkes at 350 - 400 rpm in ice. The homogenizing medium composed of 70 mM Na_2 EDTA, 300 mM sucrose and 10 mM 2-mecraptoethanol in 100 mM imidazol-HCl buffer at pH 7.2.

The $Na^+/K^+/Mg^{++}$- ATPase activity in 40 - 80 ug homogenate protein was determined in 0.5 ml reagent mixture containing 240 mM NaCl, 120 mM KCL, 20 mM $MgCL_2$ and 10 mM Na_2ATP in 100 mM imidazol-HCL buffer at 37° C and pH 7.2, for 30 min. Residual or Mg^{++}- ATPase activity was determined in a similar system from which KCL was omitted, in the presence of 0.5 mM ouabain. The reaction was terminated by the addition of 1.0 ml ice cold 10 % trichloroacetic acid. After centrifugation at 800 g for 10 min, the inorganic phosphate liberated from the substrate in the supernatant fluid was determined by the method of Fiske and Subbarow (1925). The specific activity of the enzyme was calculated as the difference in rate of phosphate release between the two reagent mixtures, per mg protein. Protein levels were determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Acetylcholinesterase.

Brain was weighted and quikly sonicated in 2 ml of 100 mM phosphate buffer at pH 7.2, using an ultrasonic power with cooling during and between each period of sonication. The temperature of the homogenate was not allowed to rise above 4°C. The homogenate was centrifuged in cold centrifuge at 22.000 g for 20 min. The supernatant was transferred to clean test tube immersed in ice bath and analysed for enzyme activity using a test combination from Boeheringer Mannheim which based upon the procedure of Ellman et al. (1961) and the use of acetyethiocholine as substrate. Brain tissue enzyme activity is expressed in terms of unit/mg protein (Lowry et al., 1951).

Cortisol determination

Plasma cortisol was determined by RIA using an antibody raised in rabbits. Plasma (20 ul) was extracted by 100 % ethanol. The extract was dried and redissolved in 500 ul 5% ethanol. The radioactivity of 100 ul of this solution was measured to determine the extraction efficiency. Then 200 ul of the dissolved extract or 200 ul standard solution ranging from 10

to 5000 pg cortisol + 100 ul (^{3}H) cortisol (4000 cpm) + 400 ul antibody dissolved in borate buffer 1:7000 was incubated on ice for one hour. Then 100 ul dextran-coated charcoal was added and the mixture centrifuged for 10 min at 4000 rpm. The supernatant was transferred to a scintillation vial and mixed with 8 ml scintillation liquid for counting in a Packard LSC.

Statistical analysis

Data were analysed using Student's test. Values are given in the Figures as mean + S.E.M. Differences between experimental and control means were considered significant when $P < 0.05$.

RESULTS

Concentration of plasma cortisol

Changes of plasma cortisol levels during exposure to DDT or atrazine are presented in Fig. 1. The results clearly show that DDT, at both concentrations, rapidly increased the hormone levels in plasma during the first 12 hr of exposure. This initial elevation was followed by decrease to a level which still significantly higher than the controls. Atrazine, at both concentrations, produced a significant dose-dependent increase of cortisol. The elevation was higher with the lower concentration (100 ug/liter).

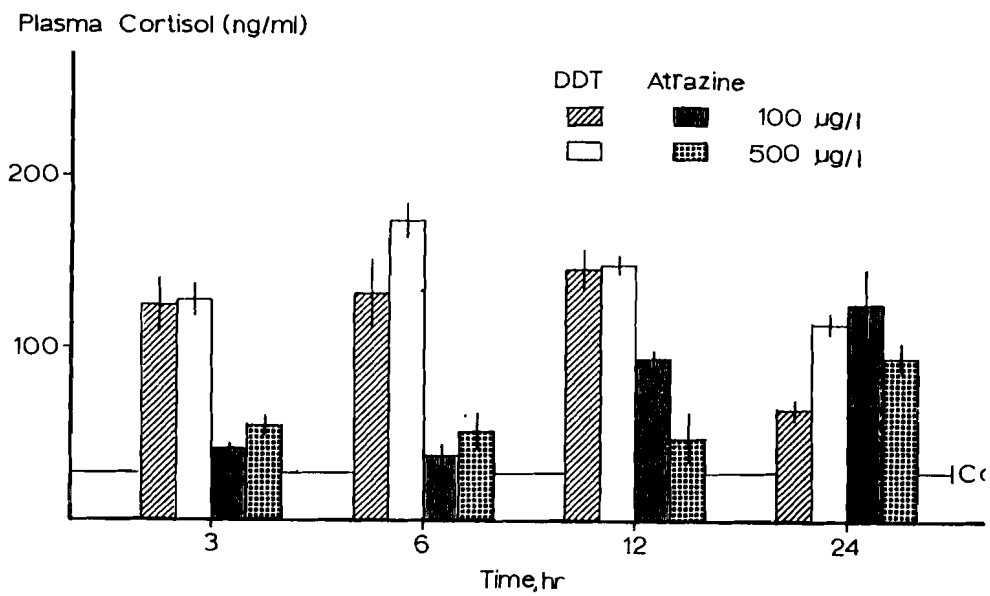

Fig. 1. Changes of plasma cortisol levels in carp exposed to two sublethal concentrations of DDT or atrazine for different lengths of time.

Gill ATPase activities

The results of in vivo effects of DDT or atrazine on the gill Na^+-K^+-ATPase activity are presented in Fig. 2. Exposure to both concentrations of DDT resulted in a rapid highly significant ($P < 0.001$) inhibition of the enzyme, recovery to nearly the controls level was observed towards the end of the experiment. Na^+-K^+-ATPase was inhibited by atrazine at both concentrations, the inhibition was greater with the higher concentration (500 ug/liter), recovery of the enzyme activity to normal control levels

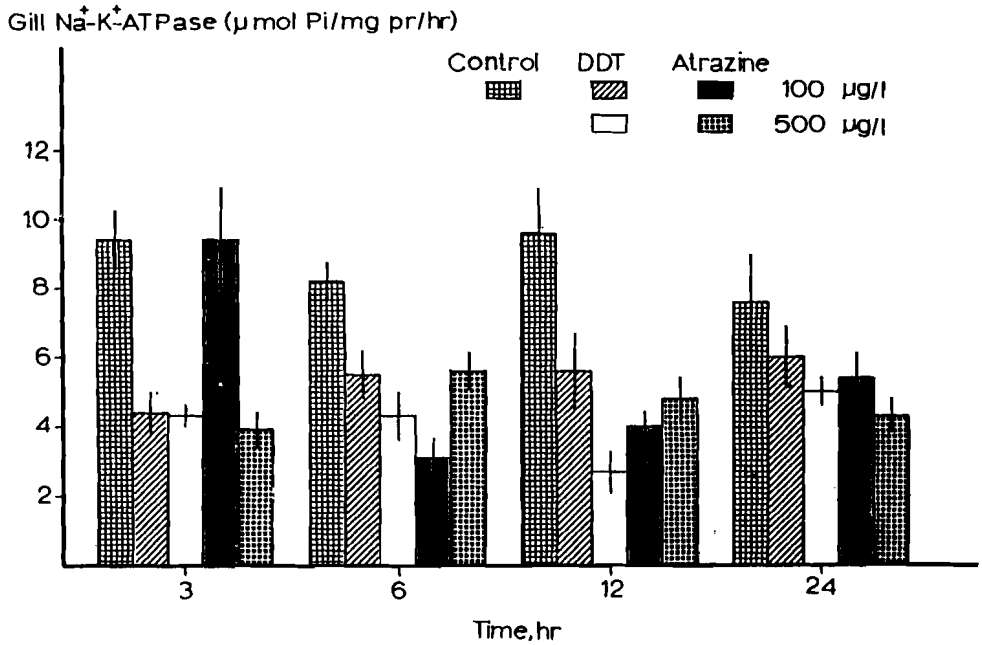

Fig. 2. Changes of gill Na^+-K^+-ATPase activity in carp exposed to two sublethal concentrations of DDT or atrazine for different lengths of time.

was recorded only with the lower concentration (100 µg/liter). Table 1 summerizes the effects of DDT and atrazine on the ouabain insensitive Mg^{2+} - ATPase. Inhibition of the enzyme was noticed in fish 12 hr after exposure to both concentrations of atrazine. Changes of the enzyme activity during exposure to both concentrations of DDT were not statistically significant.

Brain acetylcholinesterase activity

The alterations in AChE activity after treatments are presented in Table

TABLE 1
CHANGES OF GILL Mg^{2+} -ATPase ACTIVITY (μ mol Pi/mg protein/hr)

Exposure (hour)	Control	DDT (ug/l)		Atrazine (ug/l)	
		100	500	100	500
3	19.7 ± 1.7	21.5 ± 0.5	18.2 ± 1.4	21.3 ± 1.6	21.0 ± 1.7
6	18.6 ± 0.7	19.1 ± 1.1	16.5 ± 1.4	19.7 ± 2.1	19.2 ± 0.7
12	15.2 ± 1.6	13.7 ± 2.5	12.0 ± 1.4	7.8 ± 0.5*	11.5 ± 1.0*
24	20.0 ± 2.7	20.0 ± 1.4	22.2 ± 2.5	14.6 ± 0.3	19.2 ± 2.6

* $P < 0.05$

TABLE 2
CHANGES OF BRAIN ACETYLCHOLINESTERASE ACTIVITY (μg/gm protein).

Exposure (hour)	Control	DDT (ug/l)		Atrazine (ug/l)	
		100	500	100	500
3	270 ± 8.5	313 ± 21.6*	347 ± 11.8*	274 ± 11.2	286 ± 10.2
6	302 ± 15.0	250 ± 8.0*	297 ± 12.3	244 ± 17.0*	278 ± 4.0
12	326 ± 14.3	343 ± 18.0	352 ± 16.4	386 ± 7.6*	287 ± 9.3
24	295 ± 11.0	231 ± 9.5*	237 ± 16.0*	260 ± 7.3	257 ± 5.2

* $P < 0.05$

2. After 3 hr, AChE activity of fish exposed to DDT was higher than that of control fish. The enzyme was significantly inhibited by exposure hr 24. The level of the increase and that of the decrease was not dosedependent. Only with the lower concentration of atrazine an inhibition of AChE activity was measured in fish by exposure hr 6.

DISCUSSION

Elevation of plasma cortisol

In various species of teleosts, circulating corticosteriod concentrations have been shown to increase to handling (Strange el al., 1978; Barton et al., 1980), salinity changes (Assem and Hanke, 1981), oxygen deplition (Tomasso el al., 1981), environmental metal contamination (Schreck and Lorz, 1978) and pesticide contamination (Grant and Mehrle, 1973).

The resting level of cortisol in tranquil carp was 29 + 10 ng/ml. This value is in a good agreement with that recorded by Ilan and Yaron (1976). In the present study, treatment with sublethal concentrations of DDT or atrazine resulted in an increase of cortisol levels in plasma. The two compounds appear to have slightly different modes of action in eliciting a general stress response in carp. Exposure to atrazine caused a small but significant increase of cortisol, while DDT exposure was followed by a large cortisol response, strong elevation of the hormone was found after 3 hr.

The dynamics of circulating cortisol levels in exposed fish depends on the toxicant tested. The alarm phase of stress is charactrized by an immediate increase in cortisol at all levels of DDT and atrazine. This early elevation of cortisol may reflects the reaction of the fish to the recognition of the presence of a noxious or potentially harmful substance. This initial reaction phase was followed by the resistance phase, during which the fish tries to maintain or regain homeostasis. Resistance led to compensation at both concentrations of DDT but to further resistance at both concentrations of atrazine, as seen by the gradual elevation in cortisol titers throughout the rest of the test period. These different cortisol reaction between DDT and atrazine may be attributed to the possibility that DDT is more noxious than atrazine. The results indicate that cortisol elevation may have value as an early indicator of stress in carp exposed to sublethal concentrations of DDT or atrazine.

Inhibition of gill ATPase

The role of gills in maintaining a proper osmotic value and ion composition of the blood in teleosts is well established (Evans, 1980). The enzyme Na^+-K^+-ATPase is involved with the active transport of sodium across the gills (Lock et al., 1981). Reduction in the enzyme activity may inhibit the abiiity of freshwater fish to maintain a hyper-osmotic internal milieu. In the present experiments the Na^+-K^+-ATPase was very sensitive to DDT and atrazine. The alarm phase was charactrized by an inhibition of the activity.

This response lasted a few hours and was followed by the resistance phase, the enzyme activity was recorded to within the normal range. Therefore, Na^+-K^+-ATPase inhibition appear promising as an indicator of stress caused by DDT or atrazine.

The effects of DDT or atrazine on ouabain-insensitive Mg^{2+}-ATPase activity was not correlated with Na^+-K^+-ATPase responses. The enzyme activity remained more or less unchanged. The practicality of using changes in gill Mg^{2+}-ATPase activity as a general index of stress during exposure of carps to DDT or atrazine is thus limited. The fact that sublethal concentrations of DDT produce no enzyme reaction, further complicate this matter.

Brain acetylcholinesterase

Acetycholinestrase assay in fishes have proved valuable in detecting sublethal poisoning after both experimental and operational applications of anticholiestrase agents (Livingston and Goodwin, 1974; Coppage and Braidech, 1976). The influnce of DDT on the activity of brain AChE was very pronounced. The initial increase of the enzyme activity seems to represent the alarm reaction to the presence of DDT and during which more nerve impulse transfer may be needed. A similar initial increase of brain AChE activity was found in the sheepshead minnow, **Cyprinodon variegatus,** exposed to lower concentrations of the organophosphate insecticide Diazinon (Goodman et al., 1979). The response of the enzyme to atrazine was not clear as to DDT. The alarm and resistance phase could not be destinguished with the higher concentration (500 ug/liter), for the enzyme activity remained stable throughout the period of exposure.

Summarizing these results, it was found that the changes of plasma cortisol levels, gill Na^+-K^+-ATPase activity and brain AChE activity gave a good estimation for stress conditions.

The results demonstrate that it is the best way to determine the toxicity of an unknown solution by comparing the effects of two or three different concentrations with those of a known sublethal concentration (of atrazine, DDT or others) for 24 hr. In this case, the cortisol reaction is the most sensitive one. However, the enzyme reactions are much easier to investigate because each laboratory can handle the available tests without difficulties. If the laboratory is able to carry out a RIA for cortisol, this is the most recommended test.

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