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Survival, Behavioural Response and Haematological Profile of Catfish *Heteropneustes fossilis* Exposed to DDT

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Abstract Bioassay studies involving *Heteropneustes fossilis* were conducted with different concentrations of DDT. Ninety-six hour LC_{50} was found to be 2.95 ppm. Exposure of the fish to different concentrations, resulted in remarkable changes in behaviour. Erratic swimming, jerky movements and convulsions before death were evident and the severity varied with DDT concentration. Regarding the blood profile of fish exposed to sub-lethal dose of DDT for 11 days, the haematocrit and erythrocyte counts were unaltered but white blood cell-therombocyte counts (WBC-T) and leucocrit value declined markedly ($P<0.05$). Changes in differential leucocyte pictures were also observed. Data have been interpreted and haematological indicators of stress pointed out.

Haematological parameters of fishes are increasingly employed for assessment of nutritional status, vitality, pathological state, environmental stress, and above all the general well being of these animals. Use of haematology in monitoring stress levels of aquatic pollution has been amply emphasized by Bouck and Ball (1966), Eisler and Edmunds (1966), Ishihara et al. (1967), Johnson (1968), Blaxhall (1972), Hickey (1976) and McLeay and Gordon (1977).

Attempts were made by the authors to determine the effect of DDT (1,1,1-trichloro-2,2-bis-p-chlorophenyl ethane) on *H. fossilis* (Bloch), a commercially important air breathing catfish found in freshwaters of the Indo-Pacific region. Considerable quantities of DDT enter the aquatic environment due to its widespread application in agriculture and public health programmes related to control of the insect menace. Stability of this organochlorine compound leads to its accumulation in various segments of the environment. Some of the aspects studied and reported in this paper include, besides survival and behaviour, changes occurring in red blood cell counts, haematocrit, white blood cell-thrombocyte count, leucocrit, and differential leucocyte counts of fish exposed to DDT.

Materials and methods

Live specimens of *H. fossilis* (total length 19.8–22.0 cm, body weight 40–57 g) were caught from natural populations in the Aligarh ponds (27°34′30″N, 70°4′26″E) in March–April 1982. Specimens in apparently healthy condition were selected. Those showing signs of any disease and emaciation were disregarded. They were brought to the laboratory and reared in glass aquaria supplied with water (temperature 28–30°C, pH 7.1–7.3, dissolved oxygen concentration 6.5–8.0 ppm), and acclimated for a week before exposure to toxicant. The photoperiod followed the normal day light hours of Aligarh during April. During the acclimation period fish were fed daily to satiety with fresh meat chopped into fine pieces. Unused food was siphoned off and water renewed every day. Feeding was stopped 24 hours before exposing the fish to toxicant.

Toxicity tests were performed using reagent grade DDT (Wilson Laboratories, Bombay), dissolved in a small but known quantity of acetone and diluted by water. Some preliminary exploratory experiments were performed to ascertain the highest initial concentration (Low Dose) and lowest final concentration (High Dose). Final test concentrations of 2.0, 2.5, 3.0, 3.5, 4.0 and 4.5 parts per million (ppm) were then selected for exposing the fish in separate experimental glass jars of 3.0 liter capacity each. The number of specimens in each jar was 5 and the total number of fish in each concentration was 15 since 3 jars were run for a particular concentration. Control sets were also run simultaneously by taking equal numbers of specimens in equivalent volumes of DDT-free water in which the amount of acetone was mixed

in the same proportion as in the test containers. Behavioural changes in the insecticide-treated fish were observed. Mortality counts were made after every 24 hours. Dead fish were immediately removed from the test jars. Media of experimental jars were renewed daily.

The data were subjected to probit analysis (Finney, 1952). To determine the 48, 72 and 96 hours median lethal concentrations (LC_{50}), regression lines were drawn on probit graphs, with mortality percentage transformed into probits and plotted against logarithmic values of concentration after multiplying each arithmetic value by 100. The relationship between concentration (X) and probit kill (Y) was evaluated using the following regression equation:

$$Y=a+bX$$

The empirical probits and the corresponding percentage kill values were read off from the column of known dosage (first decimal place of the Log:10 value of concentration in ppm) as given by Fisher and Yates (1953).

For studying the haematological changes, fish specimens were exposed to a sub-lethal DDT concentration of 0.5 ppm for a period of 11 days during which toxicant treated water in the aquaria was renewed after daily sampling. Fish of only one sex (females) were selected for this experiment because preliminary investigations carried out earlier revealed sex-related differences in some of the haematological values (Murad, 1981). A control set was run simultaneously in which a small quantity of acetone equivalent to that in test aquaria was added. Three fish, each from experimental as well as from control sets were netted out at 24 hour intervals. Water adhering to the bodies was removed with absorbent paper within 10–15 seconds of capture.

Blood was collected in heparinized vials by severing the caudal peduncle of the fish specimens within a minute of sampling and was then transferred to a refrigerator. Samples containing clotted blood were discarded. Haematocrit and leucocrit were determined according to the methods described by McLeay and Gordon (1977). Blood was drawn in heparinized capillaries which were sealed and the content centrifuged at 6,000 rpm for 20 minutes and the haematocrit value was recorded. For leucocrit value, height of the buffy layer was measured to the nearest of 0.02 mm using an occular micrometer.

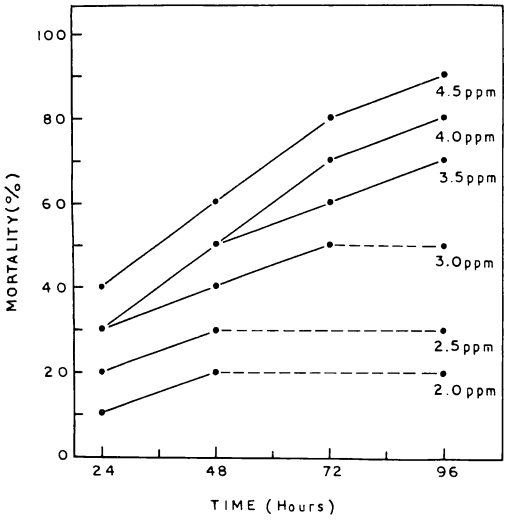
Total white blood cell (WBC)-thrombocyte (T) count and red blood cell (RBC) count were made by drawing blood into a standard pipette and by diluting 1 : 200 with Rees-Ecker diluting fluid (Klontz and Smith, 1968). The two types of cells were identified and enumerated under a microscope by the help of a Neubauer Hemocytometer.

Differential leucocyte count was carried out by the method of Pachkov (1964). The blood was drawn on clean slides, allowed to dry at room temperature, fixed in methanol and stained in Giemsa. The cells identified include lymphocytes, thrombocytes, neutrophiles and eosinophils. Two hundred cells were counted separately from the best two smears per fish under the 1,000×- oil immersion lense. Absolute counts (-/mm^3) and relative (%) means were tabulated.

Results and discussion

Behavioural changes. Exposure of *H. fossilis* to DDT at different concentrations resulted in remarkable changes in behaviour. Erratic swimming, fast jerky movements and convulsions prior to death were most evident, the severity of which paralleled the concentration of DDT. Following an initial restless condition, fishes calmed down in lower concentrations (up to 3.0 ppm) after some time, but at higher concentrations fishes showed restlessness throughout the experimental period. Opercular beats increased to 120/minute from a value of 64–70/minute obtained in control specimens. Ejection of water from the mouth was also seen quite often in solutions of higher concentrations. The color of the body became pale yellow. Barbels were the first to lose their color. Mucous was secreted profusely. Exposed specimens frequently collided with each other probably due to loss of vision sensitivity. Just prior to death fish settled at the bottom.

The observed changes in behaviour of *H. fossilis* may be due to a direct manifestation of the disturbances in physiological mechanism which according to Marler and Hamilton (1966) initiate, maintain and terminate behaviour.



Fgi. 1. Mortality of *Heteropneustes fossilis* exposed to different concentrations of DDT. Dotted lines indicate zero mortality.

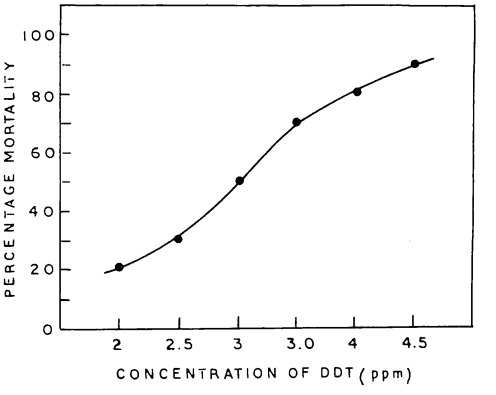


Fig. 2. Percentage mortality of *Heteropneustes fossilis* exposed to different concentrations of DDT.

Erratic swimming, jerking and convulsions observed after the exposure may be due to the effect of DDT on the central nervous system (CNS). Available evidences indicate that DDT exerts an insiduous effect on the CNS (Peakall, 1967). Signs of stimulation are referable primarily to excitation of supraspinal structures causing hyperexcitability, tremors and convulsions (Goodman and Gilman, 1975). Increased opercular movements of fishes when introduced in toxic environment implied rise in oxygen consumption. This may be due to hyperexcitability which involves considerable energy expenditure and thereby makes for greater demands of oxygen. Behavioural changes and reaction to DDT, as seen in *H. fossilis*, were also observed in *Colisa fasciatus* and *Notopterus notopterus* (Verma et al., 1974, 1975), *Labeo rohits* (Verma et al., 1977) and *H. fossilis* (Verma et al., 1978) resulting from exposure to toxicants.

Increased mucous secretion possibly tends to minimise the irritating effect of the pesticide by forming a protective coating on the skin. Damaged vision, evident in *H. fossilis*, has been reported by Alderdice and Worthington (1959) in coho salmon (*Oncorhynchus kisutch*) exposed to high concentration of DDT.

Loss of equilibrium, as indicated by abnormal swimming behaviour, might be due to the damage inflicted on the lateral line sensory cells (neuromasts) of the fish by the pesticide entering through their pores. Concordant views have been expressed by Anderson (1968) in case of *Salvelinus fontinalis*.

Mortality and LC_{50}. Results of tests with different DDT concentrations are represented in Figs. 1–2. The LC_{50} values and regression equations for various durations of exposure are presented in Table 1. During the test period no mortality was observed in the control sets. A survey of literature revealed that the LC_{50} for *H. fossilis* was higher than that reviewed for several other teleosts (Johnson, 1968; Spehar et al., 1982). This may be due to lower susceptibility of the air breathing catfish to DDT in comparison with exclusively aquatic breathing fishes.

Haematological changes. Data on selected

Table 1. Toxicity of DDT to *Heteropneustes fossilis*

Time of treatment (in hours)	Heterogeneity (Chi-square test)	Regression equation	LC_{50} (in ppm)
48	$\chi^2(4)=59.98$	$Y=-5.85+3.98X$	3.55
72	$\chi^2(4)=41.42$	$Y=-21.60+10.00X$	3.02
96	$\chi^2(4)=17.91$	$Y=-12.54+7.00X$	2.95

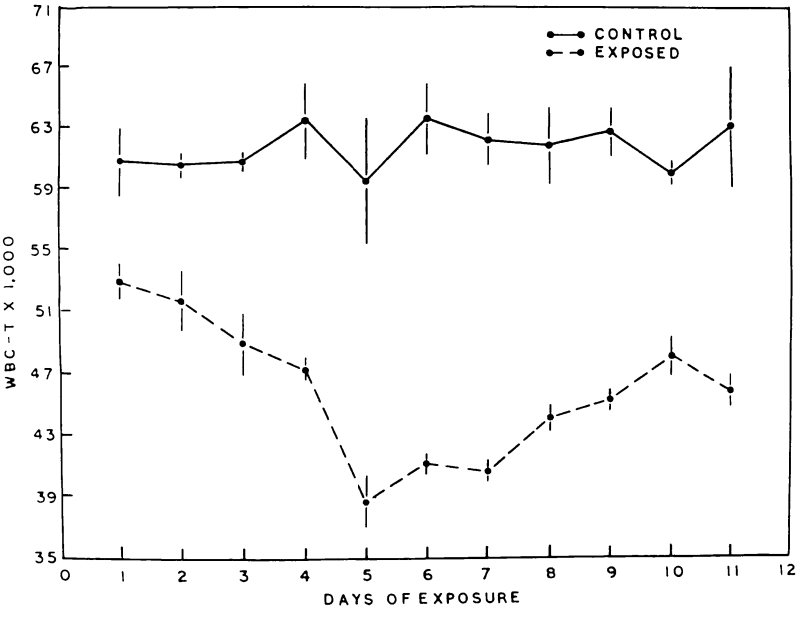


Fig. 3. White blood cell-thrombocyte count (-/mm^3) of *Heteropneustes fossilis* exposed to DDT.

Table 2. Effect of DDT exposure on RBC-count, WBC-T count, haematocrit and leucocrit in *Heteropneustes fossilis* (values are mean of 3 observations; standard error is shown in parentheses).

Days of exposure	RBC-count (million/mm^3)		WBC-T count ($\times 10^4$/mm^3)		Haematocrit (%)		Leucocrit (%)	
	Control	Exposed	Control	Exposed	Control	Exposed	Control	Exposed
1	2.31	2.21	6.06	5.29	33.90	32.92	1.19	1.03
	(0.06)	(0.01)	(0.24)	(0.12)	(0.83)	(0.54)	(0.05)	(0.04)
2	2.24	2.18	6.03	5.18	29.60	31.95	1.18	1.00
	(0.06)	(0.04)	(0.08)	(0.21)	(2.45)	(1.46)	(0.01)	(0.04)
3	2.08	2.06	6.06	4.89	30.63	29.75	1.19	0.92
	(0.05)	(0.03)	(0.38)	(0.12)	(0.72)	(1.18)	(0.08)	(0.04)
4	2.19	2.11	6.35	4.71	32.08	30.17	1.24	0.86
	(0.03)	(0.04)	(0.25)	(0.06)	(0.41)	(1.88)	(0.05)	(0.05)
5	2.20	2.19	5.95	3.86	32.31	30.76	1.17	0.70
	(0.06)	(0.01)	(0.42)	(0.17)	(0.86)	(1.11)	(0.08)	(0.03)
6	2.14	2.02	6.36	4.13	31.34	28.94	1.25	0.73
	(0.12)	(0.04)	(0.25)	(0.05)	(1.77)	(0.80)	(0.05)	(0.01)
7	2.19	2.10	6.21	4.16	32.12	29.87	1.22	0.73
	(0.10)	(0.06)	(0.18)	(0.08)	(1.54)	(1.21)	(0.03)	(0.01)
8	2.19	2.10	6.17	4.42	32.56	29.82	1.21	0.78
	(0.07)	(0.06)	(0.27)	(0.09)	(0.92)	(1.39)	(0.05)	(0.02)
9	2.16	2.19	6.29	4.54	31.63	32.44	1.22	0.87
	(0.04)	(0.04)	(0.17)	(0.02)	(0.56)	(0.99)	(0.04)	(0.01)
10	2.26	2.08	6.11	4.83	33.20	29.53	1.19	0.88
	(0.09)	(0.05)	(0.08)	(0.14)	(1.33)	(1.04)	(0.03)	(0.07)
11	2.28	2.21	6.33	4.58	33.50	31.96	1.24	0.85
	(0.08)	(0.03)	(0.41)	(0.10)	(1.17)	(0.65)	(0.08)	(0.05)

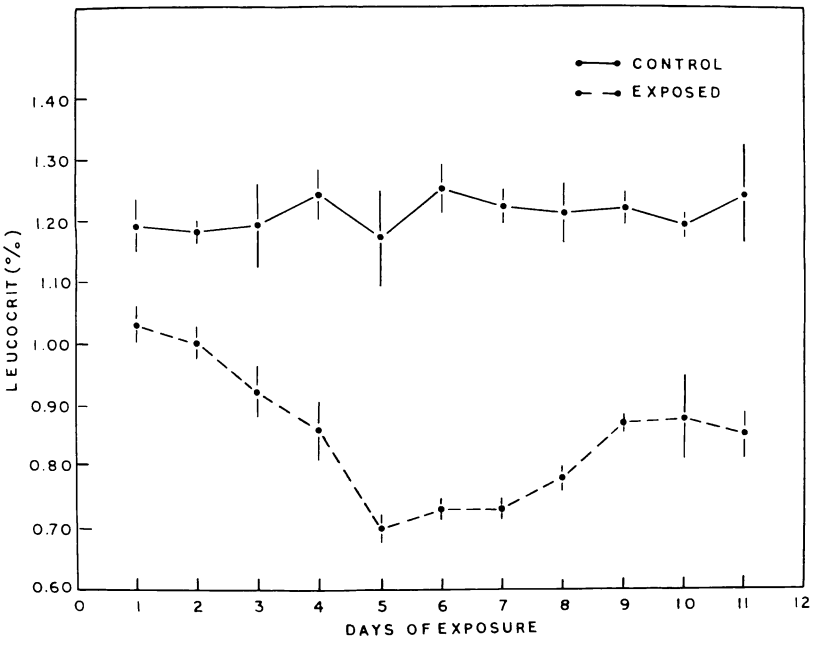


Fig. 4. Leucocrit value of *Heteropneustes fossilis* exposed to DDT.

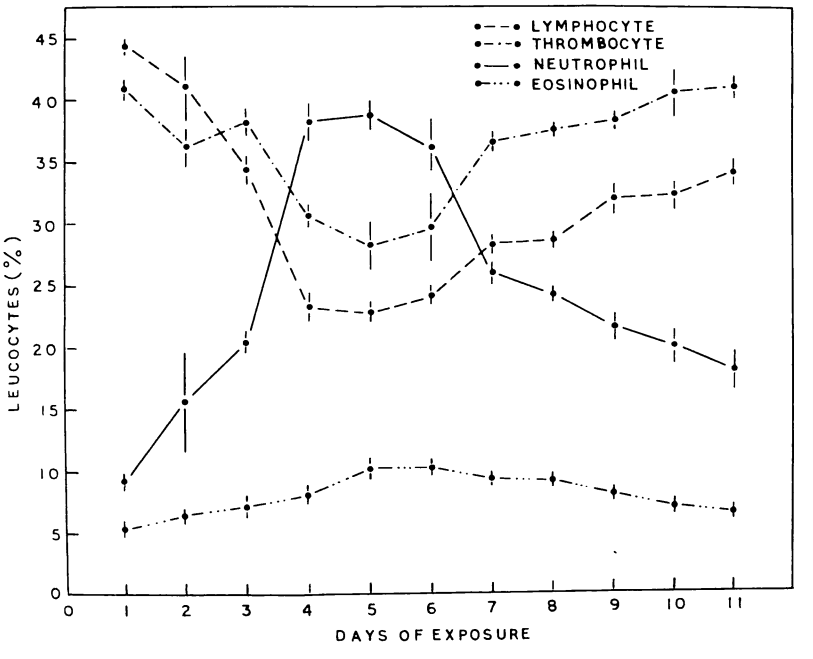


Fig. 5. Differential leucocyte count of *Heteropneustes fossilis* exposed to DDT.

haematological parameters of control specimens of *H. fossilis* and those exposed to DDT from 1 to 11 days have been tabulated (Tables 2–4) and plotted in Figs. 3–5. While haematocrit and RBC counts remained more or less unaltered during DDT exposure, significant ($P<0.05$) differences between control and exposed specimens were observed in the WBC-T and leucocrit. Both these blood parameters decreased steadily by treatment of fish with the DDT in the first five days of exposure, and after signs of gradual recovery during next four days, the values became constant. Examination of differential leucocyte pictures revealed that decrease in WBC-T count was due mainly to reduction in numbers of circulating thromobocytes and most predominantly occurring lymphocytes which outmatched rather a small numerical rise in neutrophilis and eosinophils. This haematological change can be correlated with increased

Table 3. Effects of DDT exposure on differential leucocyte count (values are mean of

Days of exposure	Lymphocyte (%)		Thrombocyte (%)	
	Control	Exposed	Control	Exposed
1	48.67 (1.33)	44.30 (0.33)	40.00 (0.58)	41.00 (0.58)
2	50.00 (1.15)	41.00 (3.05)	39.66 (0.88)	36.00 (1.76)
3	49.66 (1.76)	34.60 (1.20)	41.00 (1.15)	38.33 (1.20)
4	50.33 (1.20)	23.00 (1.53)	39.00 (1.73)	30.66 (0.67)
5	49.66 (2.19)	22.66 (0.88)	40.33 (2.19)	28.33 (2.19)
6	50.00 (1.73)	24.00 (0.57)	39.33 (1.33)	29.66 (2.19)
7	51.00 (0.58)	28.30 (0.88)	39.66 (0.33)	36.66 (0.33)
8	50.66 (0.33)	28.60 (0.67)	39.33 (1.33)	37.66 (0.33)
9	49.33 (1.20)	32.00 (1.53)	41.66 (0.88)	38.33 (0.33)
10	50.00 (2.31)	32.30 (1.20)	40.33 (3.28)	40.66 (2.03)
11	50.33 (2.03)	34.33 (1.20)	40.66 (1.76)	41.00 (1.0)

Correlation coefficient and regression equation between days (X) and relative leucocyte

1 to 5 days	Regression equation	Y=0.33X+48.97	Y=−6.13X+51.50		Y=−3.07X+44.07
	Correlation coefficient (r)	r=0.587 (n.s.)	r=−0.968 ($P<0.01$)	r=0.0	r=−0.920 ($P<0.05$)
6 to 11 days	Regression equation	Y=−0.08X+50.87	Y=1.92X+13.64	Y=0.31X+37.49	Y=1.98X+20.48
	Correlation coefficient (r)	r=−0.246 (n.s.)	r=0.966 ($P<0.01$)	r=0.645 (n.s.)	r=0.90 (n.s.)
1 to 11 days	Regression equation	Y=0.09X+49.46	Y=−0.69X+35.49	Y=0.07X+39.65	Y=0.37X+33.97
	Correlation coefficient (r)	r=0.439 (n.s.)	r=−0.323 (n.s.)	r=0.299 (n.s.)	r=0.268 (n.s.)

n.s.=Not significant.

activity of the pituitary-interrenal stress axis. That pesticides cause stress responses in fish has been documented by Schreck and Scanlon (1977). Lymphopenic response is thought to result from a stress-mediated increase in secretion of corticosteriods by the interrenal tissue (McLeay, 1973a). Yaron and Ilan (1974) observed increased levels of corticosteriods in carp treated with O'P'-DDD. The works of Dougherty (1960) and McLeay (1973b, c) make it amply clear that lymphocytes are susceptible to lysis by corticosteriods. Both stress and corticosteriod administration have been shown to result in lymphopenia in salmonids (Weinreb, 1958; McLeay, 1973b, c, 1975). In the present study, decrease in total white blood cell-thrombocyte count reflects a state of stress in fish and points to the role of DDT as a potential environmental stressor. Stress-induced lowering of the WBC-T count has been reported by Iwama

3 observations; standard error is shown in parentheses).

Neutrophil (%)		Eosinophil (%)	
Control	Exposed	Control	Exposed
7.00	9.33	3.66	5.33
(0.58)	(0.67)	(0.33)	(0.33)
7.33	15.66	3.00	6.66
(0.33)	(4.41)	(0.0)	(0.33)
5.66	20.33	3.66	7.00
(0.33)	(0.33)	(0.33)	(0.58)
7.00	38.33	3.66	8.00
(1.15)	(1.86)	(0.33)	(0.58)
6.66	38.66	3.33	10.33
(0.33)	(1.45)	(0.33)	(0.88)
7.33	36.00	3.33	10.33
(0.88)	(2.31)	(0.33)	(0.33)
6.33	26.00	3.00	9.33
(0.67)	(1.0)	(0.58)	(0.33)
7.33	24.33	2.66	9.33
(1.20)	(0.33)	(0.33)	(0.33)
5.66	21.66	3.33	8.00
(0.33)	(1.20)	(0.67)	(0.58)
8.00	20.00	2.66	7.00
(1.0)	(1.33)	(0.33)	(0.58)
6.83	18.00	3.00	6.66
(1.45)	(2.0)	(0.58)	(0.67)
counts (Y)			
$Y=-0.1X+7.03$	$Y=8.13X+0.06$		$Y=1.13X+4.06$
$r=-0.248$ (n.s.)	$r=0.960$ ($P<0.01$)	$r=0.060$	$r=0.961$ ($P<0.01$)
$Y=0.02X+6.71$	$Y=-3.16X+51.17$	$Y=-0.06X+3.48$	$Y=-0.76X+14.92$
$r=0.054$ (n.s.)	$r=-0.923$ ($P<0.05$)	$r=-0.356$ (n.s.)	$r=-0.979$ ($P<0.01$)
$Y=0.02X+6.70$	$Y=0.22X+23.08$	$Y=-0.07X+3.64$	$Y=0.12X+07.31$
$r=0.090$ (n.s.)	$r=0.075$ (n.s.)	$r=-0.645$ (n.s.)	$r=0.232$ (n.s.)

et al. (1976) in the case of *Oncorhynchus kisutsh* exposed to dehydroabietic acid and by McLeay and Gordon (1977) in the case of salmon exposed to kraft pulpmill wastes.

The thrombocytopenic response was found to be slow and less profound than the lymphopenic one in *H. fossilis* exposed to DDT. Such a slow response following the onset of stress induced by corticosteroid administration was also observed by Weinreb (1958) in *Salmo gairdneri*.

The eosinophilia noticed in *H. fossilis* could possibly represent a chemotactic response to DDT. Marked eosinophilia resulting from mercury and copper poisoning was noticed in mullet fingerlings (Helmy et al., 1978). Ringeon (1938) and Harrison (1962) reported oesinophilia in humans suffering from arsenic and phos-

Table 4. Calculated differential leucocyte-thrombocyte counts (values are mean of

Days of Exposure	Lymphocyte		Thrombocyte	
	Control	Exposed	Control	Exposed
				$\times 10^4/mm^3$
1	2.952 (0.136)	2.345 (0.034)	2.424 (0.061)	2.169 (0.052)
2	3.01 (0.051)	2.123 (0.153)	2.393 (0.062)	1.902 (0.122)
3	3.003 (0.144)	1.694 (0.031)	2.488 (0.169)	1.879 (0.093)
4	3.189 (0.086)	1.083 (0.050)	2.483 (0.168)	1.446 (0.031)
5	2.938 (0.094)	0.875 (0.043)	2.411 (0.219)	1.086 (0.036)
6	3.171 (0.014)	0.992 (0.029)	2.507 (0.145)	1.223 (0.087)
7	3.171 (0.103)	1.177 (0.028)	2.466 (0.072)	1.524 (0.032)
8	3.127 (0.124)	1.268 (0.044)	2.421 (0.042)	1.664 (0.014)
9	3.109 (0.120)	1.454 (0.057)	2.624 (0.078)	1.741 (0.006)
10	3.053 (0.100)	1.564 (0.071)	2.468 (0.182)	1.965 (0.085)
11	3.192 (0.228)	1.572 (0.051)	2.563 (0.105)	1.876 (0.006)
	Correlation coefficient and regression equation between days (Y) and absolute count			
1 to 5 days	Regression equation	Y=146.47X +2975.2	Y=−3879.44X +27875.5	Y=65.4X+24200.4	Y=2622.19X+ +24830.4
	Correlation coefficient (r)	r=0.231 (n.s.)	r=−0.962 ($P<0.01$)	r=0.238 (n.s.)	r=−0.968 ($P<0.01$)
6 to 11 days	Regression equation	Y=−77.1X +32027.3	Y=1214.2X +3058.2	Y=140.3X +23888.9	Y=1332.8X +5326.6
	Correlation coefficient (r)	r=−0.281 (n.s.)	r=0.977 ($P<0.01$)	r=0.354 (n.s.)	r=0.935 ($P<0.02$)
1 to 11 days	Regression equation	Y=161.8X +29864.8	Y=−558.12X +18026.9	Y=121.7X +24040.2	Y=−68.5X +17206.7
	Correlation coefficient (r)	r=0.562 (n.s.)	r=−0.402 (n.s.)	r=0.584 (n.s.)	r=−0.069 (n.s.)

n.s.=Not significant.

phorus intoxication and supported the view that this phenomenon in blood is a reaction against invasion of a wide variety of toxic products.

Increase in WBC-T count, leucocrit and lymphocyte-thrombocyte percentage following their all-time low value on fifth day of exposure of the fish signifies identity with stress-related changes elaborated by Selye (1950, 1971). The lymphopenic response is similar, both during the alarm reaction to stress and during the subsequent stage of resistance to stress (recovery).

The study of specific haematological parameters particularly WBC-T counts, leucocrit and differential leucocyte counts of fish exposed for short period to environmental stressors including DDT may provide a rapid and sensitive method for predicting the effects of sublethal

3 observations each, standard error is indicated in parentheses).

Neutrophil		Eosinophil	
Control	Exposed	Control	Exposed
0.425	0.494	0.223	0.282
(0.034)	(0.032)	(0.019)	(0.014)
0.442	0.808	0.181	0.344
(0.017)	(0.188)	(0.002)	(0.009)
0.343	0.995	0.222	0.345
(0.025)	(0.026)	(0.021)	(0.033)
0.443	1.809	0.231	0.377
(0.057)	(0.087)	(0.011)	(0.019)
0.397	1.497	0.201	0.402
(0.031)	(0.098)	(0.028)	(0.042)
0.468	1.489	0.213	0.427
(0.052)	(0.093)	(0.024)	(0.011)
0.391	1.080	0.186	0.388
(0.027)	(0.032)	(0.027)	(0.017)
0.457	1.075	0.165	0.413
(0.070)	(0.016)	(0.018)	(0.010)
0.356	0.984	0.208	0.364
(0.010)	(0.044)	(0.031)	(0.022)
0.426	0.966	0.163	0.338
(0.045)	(0.060)	(0.015)	(0.022)
0.396	0.826	0.192	0.306
(0.072)	(0.080)	(0.038)	(0.029)
(-/mm^3) (X).			
Y=−55.5X+4268.4	Y=2561.4X+3519.8	Y=6.4X+2095.3	Y=271.8X+2681.8
r=−0.210 (n.s.)	r=0.765 (n.s.)	r=0.050 (n.s.)	r=0.957 (P—0.02)
Y=−102.0X+5024.3	Y=1071.1X+1980.7	Y=−37.8X+2197.5	Y=−229.1X+5672.1
r=−0.447 (n.s.)	r=−0.890 (P—0.05)	r=−0.33 (n.s.)	r=−0.939 (P<0.02)
Y=−13.72X+4214.3	Y=−6.1X+10966.7	Y=−37.9X+2212.5	Y=19.4X+3505.2
r=−0.113 (n.s.)	r=−0.006 (n.s.)	r=−0.539 (n.s.)	r=0.144 (n.s.)

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DDT に曝露した *Heteropneustes fossilis* の生存，行動および血液性状

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種々な濃度の DDT に曝露した．96 時間 LC_{50} は 2.95 ppm であった．曝露後には行動の異常が顕著であった．遊泳不全，痙攣様運動および痙攣が死ぬ前に現われ，DDT 濃度が高いほど重篤であった．致死濃度以下の濃度に 11 日間曝露した後の血液性状は，ヘマトクリット値と赤血球数は不変であったが，白血球一種球数と白血球容積は著しく低下した．白血球組成にも変化がみられた．得られた結果の解釈とこの結果がストレスによるものであることを指摘した．