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Diazinon Induced Changes in the Serum Proteins of Large Mouth Bass, *Micropterus salmoides*

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Diazinon [O,O-Diethyl O-(2-isopropyl-6-methyl-4-pyrimidinyl) phosphorothioate] is a broad spectrum organophosphorus pesticide extensively used all over the world to control flies, cockroaches, lice on sheep, insect pests of ornamental plants and food crops (almonds, corn, apples and tobacco), nematodes, soil insects in lawns and croplands (Smith 1993). Smith (1993) reported that the annual acreage planted with diazinon treated seeds in the U.S. is estimated to be 1,150,000.

Toxicants, including diazinon, can enter the bloodstream through the gills or the gastrointestinal tract (Doving 1991). Thus, evaluation of fish blood provides valuable information concerning the physiological response of fish to changes in the external environment, including the presence of toxicants such as diazinon. (Van Vuren et al., 1994). Electrophoretic determination of the serum proteins of dying fish may provide legal evidence in suspected pollution cases (Brooke 1964). Thus, this study employs the electrophoresis and densitometer readings of SDS-PAGE gel to analyze the effect of diazinon on the serum proteins of largemouth bass *(Micropterus salmoides).*

MATERIALS AND METHODS

The detailed methods of collection, acclimation and exposure of fish are explained in Pan and Dutta, 1998. Prior to blood sampling, five fish from each group were anesthetized with tricaine methane sulfonate (MS-222) using 100 mg/L tap water. Blood samples of 0.3 to 0.6 mL were obtained by direct cardiac puncture through the base of the heart. The heart was exposed by a midline incision and the pericardium was partially removed. This method is preferred over the caudal vessel method since it yields a larger blood sample. The sampling of blood was performed in May 1996 to avoid seasonal variations of serum proteins. The collected blood was allowed to clot completely for one hour at room temperature. These blood samples were centrifuged for 15 minutes at 3600 RPM to separate the serum from the cells. The supernatant was pipetted from the centrifuge tube and transferred to a microcentrifuge tube. The samples were stored for two weeks at -20° C before the electrophoretic study was conducted.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) with a vertical, discontinuous buffer Mini-PROTEAN II Cell/PowerPac 300 System was

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employed to analyze the fish serum proteins. The electrophoretic methodology used in this study was modified from Richmonds and Dutta (1992).

Five µL serum sample was added into a microcentrifuge tube. In the tube, 10 µL 2X sample buffer was added and mixed well. The high and low range molecular weight marker standard proteins are provided by BIO-RAD Laboratories specific for SDS-PAGE silver stain. High and low molecular weight (MW) markers each 5 µL were prepared and added into microcentrifuge tubes. Ten µL 2X sample buffer was then added to each. The sample tubes and MW marker tubes were boiled for 5 minutes in water bath.

Fifteen µL of each sample or MW marker solution in sample buffer was added into each lane. The remaining lanes were loaded with 15 µL blank sample buffer.

Electrophoresis was conducted at 200 volts for 1 to 1.5 hour by watching the movement of the tracking dye. The power was then turned off. The plates were separated by the spacer. The stacking gel was removed and one of the gels was marked. Silver stain was used to stain the gels (Pan 1996).

Paired sample t-test was used to analyze the result of each part of this study using SPSS/PC$^+$ V4.01 computer statistical analysis program.

RESULTS AND DISCUSSION

A representative silver-stained SDS-PAGE gel is shown in Figure 1. The first and last lanes are low and high molecular weight markers, respectively. The second lane, from right to left are control 90, 180, 270, 360, 450 µg/L groups. The silver staining electrophoretic patterns of SDS-PAGE gels of the control group and of the 450 µg/L group are considerably different. The absorbance curves of the UltraScan XL densitometer and GelScan XL (2.1) recordings of control, 90, 180, 270, 360, 450 µg/L diazinon exposure groups are shown in Figure 1 (Pan 1996). There were five gels from five fish of each group, and two measurements for each gel were taken. The mean values and standard errors of major serum protein fractions are listed in Table 1. Statistical analysis of major serum protein fractions is shown in Table 2. The 2-tail probability statistics of paired sample T-tests less than .05 were considered to be statistically significant. The results showed that fraction 1 and fraction 3 did not display any significant changes. However, there was a considerable quantitative increase in fraction 2 of 270 µg/L and 450 µg/L groups compared to the control group. Also, fraction 4 of all the diazinon exposed groups, except the highest diazinon exposure 450 µg/L group, showed a significant decrease compared to the control group. Fraction 5 of 270 µg/L exposure group and fraction 6 of 360 µg/L and 450 µg/L exposure groups showed significant decreases compared to those of the control group. A new serum protein fraction 7 appeared in 180 µg/L, 270 µg/L and 360 µg/L diazinon exposed groups.

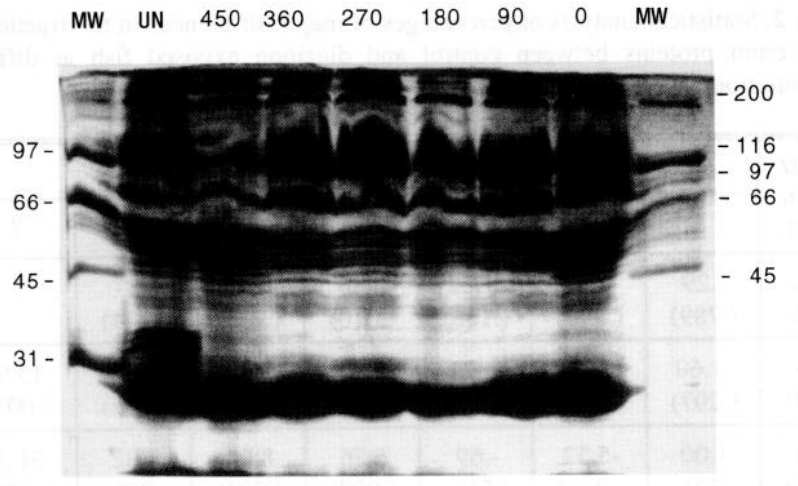

Figure. 1. Silver-stained SDS-PAGE gel of control (0 µg/L) and experimental (90-450 µg/L) fish serum proteins (values of molecular weight markers are in KD).

Table 1. UltraScan XL densitometer readings of percentages of major serum protein fractions in total serum proteins in control and diazinon exposed fish;

Protein fraction	Diazinon Exposure Concentration (µg/L)					
	0	90	180	270	360	450
1	5.210 (.108)	5.225 (.085)	5.500 (.178)	5.075 (.085)	5.151 (.155)	5.400 (.108)
2	18.350 (.185)	19.575 (.893)	18.675 (.210)	21.625 (.704)	18.225 (.304)	21.500 (.906)
3	9.902 (.255)	11.425 (.891)	11.125 (.963)	10.200 (.248)	11.301 (.892)	12.400 (1.119
4	27.600 (.460)	22.675 (.617)	23.925 (.392)	18.675 (.924)	20.875 (.388)	28.775 (.818)
5	11.302 (.432)	12.600 (.642)	10.925 (.994)	5.800 (.442)	13.000 (.742)	12.400 (.947)
6	27.550 (.499)	27.850 (.659)	26.075 (.654)	26.151 (.645)	14.225 (.287)	18.800 (.469)
7	/	/	2.600 (.187)	14.350 (.459)	18.400 (.535)	/

Mean and S.E. (in parentheses) are shown; n=5 gels with two measurements for each.

Table 2. Statistical analysis of percentages of major differences in the fractions in total serum proteins between control and diazinon exposed fish at different concentrations;

Diaz Con µg/L	Major Serum Protein Fractions						
	1	2	3	4	5	6	7
0 v 90	-.29 (.789)	-1.45 (.243)	-1.82 (.166)	5.86 (.010)	-1.48 (.235)	-.37 (.734)	/
0 v 180	-1.60 (.207)	-.95 (.413)	-1.21 (.311)	5.10 (.015)	.27 (.802)	3.06 (.055)	-13.90 (.001)
0 v 270	1.00 (.391)	-5.52 (.012)	-.69 (.542)	6.76 (.007)	8.04 (.004)	1.32 (.280)	-31.25 (.000)
0 v 360	.26 (.813)	.35 (.748)	-1.69 (.189)	15.97 (.001)	-1.66 (.195)	22.04 (.000)	-34.37 (.000)
0 v 450	-2.19 (.116)	-3.81 .032	-2.29 (.106)	-2.49 (.088)	-1.90 (.154)	51.23 (.000)	/

T statistics and 2-tail probabilities (in parentheses) of paired T-tests are shown; Number of cases=5 X 2 = 10. Diaz, Diazinon; Con, concentrations; 0 v, Control verses.

This study analyzed the major serum protein fractions of control and diazinon exposed largemouth bass. Six major protein fractions were separated by SDS-PAGE and grouped by gel scan program in the control fish. Bouck and Ball (1965) also isolated six fractions in largemouth bass in their lab, using electrophoresis system with analytical scanner. In this study, fraction 3 and 4 seem to have subfractions which were separated too poorly to be evaluated individually. Fraction 1 and 3 of diazinon exposed fish did not show any significant difference from the control fish. Fraction 2 of 270 µg/L and 450 µg/L diazinon exposed groups showed significant increases from that of the control group. Fraction 2 (low mobility protein) of diurnal oxygen pulse stressed largemouth bass in the study of Bouck and Ball (1965) also had a mean value of 26.30 while their control had a mean value of 17.12 displaying a significant increase.

The general increases in the low mobility proteins in the OP pesticide exposed fish serums were also observed by Dutta et al. (1992), Richmonds and Dutta (1992), and Datta-Munshi et al. (1999) after 24-hour exposure to malathion. The investigators suggested that the low-mobility proteins included globulin (antibodies) (Richmonds and Dutta 1992; Menzel 1970). The formation of immunoglobulin seems to occur as an immune response in the OP pesticide exposed fish. One of the reasons for the

increase in fraction 2 may be the rapid binding of pesticides to blood proteins after entering their circulatory system (Plack et al. 1979). The binding of the pesticide to the proteins may trigger some changes in the characteristics of these proteins. The changed proteins may be recognized as foreign bodies by the immune system resulting in the increased quantity of fraction 2 (immunoglobin) (Richmonds and Dutta 1992).

Damage to the gill epithelium induced by diazinon was recently observed (Dutta 1996: Dutta et al. (1996; 1998). The lesion to the gill epithelium may result in infection, inducing an immune response that will increase the quantity of fraction 2 (the globulin). Dutta et al. (1996) observed immunological changes with increases in the number of macrophages and leucocytes after *Heteropneustes fossilis* was exposed to malathion. All these findings support the electrophoretic results in fraction obtained from the diazinon exposed largemouth bass. Fraction 4 showed a significant decrease compared to that of the control group (except the 450 µg/L group). Fraction 5 of 270 µg/L group as well as fraction 6 of 360 µg/L and 450 µg/L exposure groups showed significant decreases compared to the control group.

Bouck and Ball (1965) also demonstrated that with largemouth bass, decreases in fractions 4, 5, 6 with the last two statistically significant. Fraction 4, 5, and 6 are high mobility proteins. Dutta et al. (1992); Richmonds and Dutta (1992) and Datta-Munshi et al. (1999) also reported decreases in the high-mobility proteins when fish were exposed to OP pesticides for 24 hours. The above authors suggested that the high-mobility proteins include albumin. The fish that were exposed to diazinon experienced severe stress, which could be a non-specific stress reaction. This correlates with the finding of this study, in which there was a decrease in fraction 4 (albumin). The decrease in this fraction can be explained on a functional basis. The albumin has been reported to be an osmoregulator of blood volume, an easily available protein reserve, and a transport protein (Andersson 1979). The hyperactivity caused by this pesticide may lead to the utilization of this easily accessible protein reserve fraction containing ablumin, resulting in a decreased quantity (Dutta 1996). Another possible reason for the lowered amount of albumin may be a decreased albumin synthesis in the liver because of observed severe necrosis in the heptocytes of *Heteropneustes fossilis* exposed to malathion (Dutta et al. 1993).

Interestingly, a new serum protein fraction 7 appeared in 180 µg/L, 270 µg/L, 360 µg/L groups. Dutta et al. (1992) also found a new protein fraction when Indian catfish were exposed to malathion for 48 hours. Formation of the new protein may be attributed to the cellular damages caused by this pesticide. Tissue damage would result in “leakage” from the plasma membrane of cellular proteins, for instance, intracellular enzymes, into the blood. This may also explain why fraction 7 appeared in this study.

Finally, Marinovich et al. (1994) found that diazinon could induce a dose-related inhibition of protein synthesis in HL60 cells at 24 hour exposure. This is another example of inhibition of protein synthesis by diazinon. The inhibition of protein

synthesis may cause fractions 4, 5, and/or 6 to decrease (observed in this study). It is proposed that in largemouth bass exposed to diazinon, tissue necrosis leads to losses of intracellular enzymes or other proteins. This may trigger the cells in the fish body to compensatorily repair the damaged cell organelles and to regenerate the tissue by producing greater amount of proteins, possibly resulting in the increase or decrease of certain fractions.

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REFERENCES

Andersson LO (1979) Transport proteins. In: Blomback B, Hanson LA (eds) Plasma Proteins, John Wiley and Sons, New York, p 44-54

Bouck GR, Ball RC (1965) Influence of a diurnal oxygen pulse on fish serum proteins. Trans Am Fish Soc 94:363-370

Brooke HE (1964) A review of variations found in fish serum proteins. New York. Fish and Games J 11:47-57

Datta-Munshi JS, Dutta HM, Singh NK, Roy PK, Adhikari S, Dogra JV, Ali MM (1999) Effect of Malathion, and organosphophorus pesticide on the serum proteins of *Heteropneustes fossilis* (Bloch). J Environ Path Toxicol Oncol 18:79-83

Doving KB (1991) Assessment of animal behaviour as a method to indicate environmental toxicity. Comp Biochem Physiol 100C:247-252

Dutta HM (1996) A composite approach for evaluation of the effects of pesticides on fish. In: Munshi JSD, Dutta HM (eds) Fish Morphology:Horizon of New Research, Lebanon, New Hampshire Science Publishers Int and Oxford IBH, India, p 249-269

Dutta HM, Dogra JVV, Singh NK, Roy PK, Nasar SST, Adhikari S, Munshi JSD, Richmonds C (1992) Malathion induced changes in the serum proteins and hematological parameters of an Indian catfish *Heteropneustes fossilis* (Bloch). Bull Environ Contam Toxicol 49:91-97

Dutta HM, Adhikari S, Singh NK, Roy PK, Munshi JSD (1993) Histopathological changes induced by malathion in the liver of freshwater catfish, *Heteropneustes fossilis* (Block). Bull Environ Contam Toxicol 51:895-900

Dutta HM, Munshi JSD, Roy PK, Singh NK, Adhikari S, Killius J (1996) Ultrastructural changes in the respiratory lamellae of the catfish *Heteropneustes fossilis* after sublethal exposure to malathion. Environ Poll 92:329-341

Dutta HM, Roy PK, Singh NK, Adhikari S, Munshi JSD (1998) Effect of sublethal levels of malathion on the gill of *Heteroporeustes fossilis* Scanning electron microscopic study J Environ Path Toxicol Oncol 18:79-83

Marinovich M, Guizzetti M, Galli CL (1994) Mixtures of benomyl, pirimiphos-methyl, dimethoate, diazinon and azinophos-methyl affect protein synthesis in HL-60 cells differently. Toxicology 94: 173-185

Menzel BW (1970) An electrophoretic analysis of the blood proteins of the subgenus Luxilus *(Notropis cyprinidae).* Ph.D. dissertation, Cornell University

Pan G (1996) Effects of Diazinon on juvenile largemouth bass. M.S. Thesis Kent State University

Pan G, Dutta HM (1998) The inhibition of brain acetylcholinesterase activity of juvenile largemouth bass *Micropterus salmoides* by sublethal concentrations of diazinon. Environ Res 79:133-137

Plack PA, Skinner ER, Rogie A, Mitchell AI (1979) Distribution of DDT between the lipoproteins of serum protein. Comp Biochem Physiol 62C: 119- 125

Richmonds C, Dutta HM (1992) Variations produced by malathion on the serum protein fractions of bluegill sunfish, *Lepomis macrochirus.* Comp Biochem Physiol 100C:523-526

Smith GJ (1993) Toxicology and pesticide use in relation to wildlife. In: Organophosphorus and Carbamate Compounds, CRC Press, Florida, p l-10.

Van Vuren JH, Van der Merwe M, du Preeze HH (1994) The effect of copper on the blood chemistry of *Clarias gariepinus* (Clariidae). Ecotoxicol Environ Safety 29:187-199