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The effects of the toxins of *Lophopodella carteri* (ectoprocta) on the blood gas properties of the bluegill *Lepomis macrochirus*

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THE EFFECTS OF THE TOXINS OF LOPHOPODELLA CARTERI
(ECTOPROCTA) ON THE BLOOD GAS PROPERTIES OF
THE BLUEGILL LEPOMIS MACROCHIRUS

A THESIS

PRESENTED TO THE FACULTY OF THE GRADUATE SCHOOL
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DEGREE OF MASTER OF SCIENCE

BY

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(ECTOPROCTA) ON THE BLOOD GAS PROPERTIES OF
THE BLUEGILL LEPOMIS MACROCHIRUS

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ABSTRACT

Lepomis macrochirus (bluegill) were exposed, in small containers, to homogenates of Lophopodella carteri, an ectoproct that contains substances toxic to gilled vertebrates. Blood pH, pCO_2 , and pO_2 were determined with a blood gas analyzer. Blood carbonic anhydrase activity was measured manometrically.

The ectoproct toxins caused significant decreases in blood pH and pO_2 and a significant increase in blood pCO_2 and carbonic anhydrase activity. It is proposed that these changes in blood properties were initiated by a film of mucus covering the gill epithelium. The secretion of mucus is thought to be a response to irritation of the gills by the ectoproct toxins.

INTRODUCTION

Lophopodella carteri is a freshwater ectoproct (family Lophopodidae) that was first reported by Rogick (1957) to contain a substance toxic to fishes. The first report of L. carteri in Virginia was made by Tenney and Woolcott (1962). The toxic characteristic of the organism was also noted by Tenney and Woolcott (1964) who observed that fishes died when placed in small containers that contained crushed colonies of L. carteri. Although the ectoproct grows abundantly in hatchery ponds near Stevensville, Virginia, none of the fishes in the ponds was affected by the toxins. Fish kills were noted, however, when the fishes were concentrated for collecting in the concrete spillways between ponds (Personal communication, W. S. Woolcott, 1974).

Meacham and Woolcott (1968) stated that the toxins were present in the cells of the body tissues and not in the coelomic cavity of the ectoproct as was once assumed by Oda (1958). Isolation and identification of the toxic agent have been reported by Dolan (1971) and Smith (1972). Dolan showed the toxic component to be a multi-component lipid. Smith demonstrated that the most toxic substance of the tissues of L. carteri was a lipoprotein.

Tenney and Woolcott (1964) demonstrated a close relationship between susceptibility to the toxins and a functional vertebrate gill-breathing apparatus. The toxins have also been shown to cause extreme destruction of the gill epithelium of the marbled salamander (Ambystoma opacum) (Collins et al., 1964) and of the goldfish Carassius auratus (Collins et al., 1966). Although Meacham and Woolcott (1968) found no histological damage to the gills of Gambusia affinis (mosquito fish) or the goldfish, they proposed that the toxins act on gill tissues. They suggested that the toxins inhibited the secretion of carbonic anhydrase from gill acidophilic cells. Depression of this enzyme system would lead to a buildup of blood CO₂ which would result in acidosis. They also presented a second hypothesis that the toxins have a direct inhibitory effect on metabolism where they block the electron transport in the respiratory chain.

The above studies strongly suggest that the main site of action of the toxins is gill tissue. If this is true, there should be changes in blood gas properties. The present study was designed to determine whether changes in pH, pCO₂, pO₂, % O₂ saturation and carbonic anhydrase activity of the blood of the bluegill, Lepomis macrochirus, are affected by exposure of the organism to homogenates of L. carteri.

MATERIALS AND METHODS

Bluegills were selected from the brood stock at the Virginia Fish Cultural Station located at Stevensville, Virginia in October and November, 1973 and January, 1974. They ranged in standard length (Blair et al., 1968) from 13.6 - 23.0 cm and weighed 110 - 325 g. The fish were kept in large well-aerated tanks prior to use. Colonies of L. carteri were collected at the same location in September and October, 1973. Excess water and debris were removed and the specimens were frozen and stored in Pyrex test tubes at -12 C. Prior to experimental use, homogenates of thawed colonies were prepared using a glass homogenizer.

A preliminary study was performed to determine the lethal dose of L. carteri homogenate. One bluegill was placed in each of four 10 l styrofoam buckets. Each bucket contained a total volume of 5 l of tap water drawn ten hours prior to use to eliminate chlorine and to reach room temperature (20 - 22 C). Concentrations of homogenates used in three of the buckets were: 5 g/l, 10 g/l, 20 g/l. The fourth bucket was used as a control and contained no homogenate. Results of this study indicated that a homogenate concentration of 5 g/l was adequate to produce severe respiratory distress in bluegills within two hours.

The experimental design for the main study was similar to that of the preliminary study. For each of the ten trials, two bluegills were used as controls and two were exposed to 5 g/l of homogenate in a total volume of 5 l. In all trials the fish were allowed to remain in the containers until the experimental fish displayed severe signs of distress, i.e. immobility, failure to respond to touch, and cessation of opercular movement. In all cases these distress symptoms occurred within two hours.

Dissolved oxygen and pH were periodically measured in the test chambers. The average pH of the control media was 6.43 with a mean O_2 concentration of 2.5 mg/l. Experimental buckets containing L. carteri had an average pH of 6.41 and a mean O_2 concentration of 4.5 mg/l. A Yellow Springs Oxygen Meter (model 51A) was used to determine O_2 concentration and a Corning Digital 109 pH Meter was used to determine pH.

Following the period of exposure, the fish were removed from the container, immobilized and blood was withdrawn from the heart using the method illustrated in Fig. 1. A 3 ml syringe, fitted with a 22 gauge needle, containing 0.2 ml of sodium heparin (1000 units/ml) was used in collection. One-tenth milliliter of the heparin solution was injected into the heart prior to withdrawal of blood to prevent clotting. A total volume of 1.5 ml of heparinized blood was obtained. Immediately following extraction

of blood, the syringes were sealed and placed on ice. Clotted samples were eliminated from further analysis.

Determinations of blood pH, pCO_2 and pO_2 were made within one hour following withdrawal using an IL 213-03 pH/Blood Gas Analyzer at a temperature of 37 C. All values were corrected to 22 C according to Bradley et al., (1956). Bicarbonate concentration was determined mathematically using the following Henderson-Hasselbalch equations:

$$1) \text{ pH} = \text{pK}_a + \log_{10} \frac{\text{HCO}_3^-}{\alpha \text{ pCO}_2}$$

$$2) \text{ pH} = \text{pK}_a + \log_{10} \frac{\text{HCO}_3^-}{\text{H}_2\text{CO}_3}$$

where α in equation 1 is the proportionality constant (0.03) which when multiplied by pCO_2 will give the equivalent H_2CO_3 concentration in mEq/l (Gambino, 1963). The pK in all cases is the pH (6.1) where H_2CO_3 is half dissociated (Severinghaus et al., 1956). The % O_2 saturation for blood samples was determined using the oxygen dissociation curve for bluegill (Manwell et al., 1963). The effects of changes in pH and pCO_2 on the % O_2 saturation were mathematically determined and graphically illustrated using the method of Altman and Dittmer (1971).

Blood carbonic anhydrase activity was determined using a Gilson Student Respirometer. The procedure used was a modified version of Waygood (1955). Two-tenths milliliter of fish blood from the syringes was placed in each reaction flask which contained 2 ml of Holtfreter's solution (Rugh, 1962). Powdered sodium heparin at a concentration of

10 mg/ml was added to the solution prior to the addition of blood to prevent clotting. The side arm of each flask contained 1.0 ml of .08M NaHCO₃ which was dumped into the blood medium following an equilibration of 15 minutes at 22 C. Readings of ul CO₂ were then taken every 5 minutes for 30 minutes. Calculations of ul CO₂/ml of blood /hr were based on a 15 minute interval as no change occurred after this time period.

All results were statistically analyzed using an analysis of variance test for unpaired groups (Steel and Torrie, 1960). Calculations were performed using a Wang 500 Series calculator. Differences were considered significant at the ninety five per cent level of confidence.

RESULTS

Table 1 compares the standard lengths of the 40 bluegills used in this study. The control group (\bar{X} , 17.4 cm) and the experimental group (\bar{X} , 16.9 cm) did not differ significantly (Table 2).

Table 3 presents the effects of a 5 g/l homogenate of L. carteri on the behavior of the bluegill. The general sequence of behavior observed was as follows: widely gaping opercles, with mucus excretion from that region; infrequent surfacing; snapping movements of the mouth; body listing with an apparent decrease in rate and amplitude of opercular movement. These behavioral responses were followed by

infrequent violent swimming and leaping actions after which there was listing, a cessation of opercular movement with large quantities of mucus covering the gills. Death was apparent at the end of two hours.

Table 4 reports the effects of homogenates of L. carteri on blood gas properties of L. macrochirus. The mean pH of the experimental group (7.16) was significantly lower than the mean (7.48) of the control group (Table 5). The mean $p\text{CO}_2$ of the experimental group (10.45 mmHg) was significantly higher than that of the control group (8.62 mmHg) (Table 5). In contrast, the mean concentration of HCO_3^- was significantly lower in the experimental group (3.32 mEq/l) than in the control group (5.32 mEq/l) (Table 5). Table 6 shows the ratio of the blood buffers H_2CO_3 to HCO_3^- for the experimental group (11:1) and for the control group (20:1). The toxins of L. carteri also produce a significant lowering of the partial pressure of oxygen in bluegill blood (experimental \bar{X} , 32.09 mmHg; control \bar{X} , 43.66 mmHg) (Table 7). Similarly, the L. carteri toxins caused a significant reduction in blood oxygen saturation (experimental \bar{X} , 45.0%; control \bar{X} , 61.0%). An oxygen dissociation curve indicated the Bohr and Root effects in the blood of the bluegill (Fig. 2).

The effects of toxins of L. carteri on L. macrochirus blood carbonic anhydrase activity are presented in Table 8.

The activity of this enzyme in the L. carteri treated fishes (\bar{X} , 187 ul CO₂/ml blood/hr) was significantly higher than the activity in the untreated controls (\bar{X} , 140 ul CO₂/ml blood/hr) (Table 9).

DISCUSSION

The pattern of responses of bluegill to homogenates of L. carteri was similar to that reported by Tenney and Woolcott (1964). However, they observed that violent swimming and leaping were initial responses and in the present study, these occurred only after exposure times of 75-105 minutes. Although fishes in the present study were larger than those used by Tenney and Woolcott, the difference in timing of responses has been shown to be unrelated to size (Tenney and Woolcott, 1964 and Collins et al., 1966). An explanation for the difference is probably due to different concentrations of toxins used in the two studies. At present, there is no means of measuring toxin concentrations in homogenates of L. carteri.

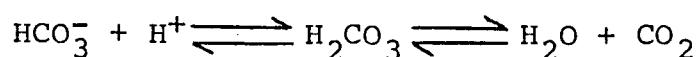
The changes in blood properties of bluegills was attributed to the accumulation of large quantities of mucus covering the gill surfaces, which occurs soon after exposure to homogenates of L. carteri. Tenney and Woolcott (1964) observed mucus secretions exuding from the mouth and opercles of several species of freshwater fish after exposure to the toxins L. carteri. Collins, et al., (1966) over exposed pharyngeal surfaces of C. auratus and larval

Ambystoma opacum. The release of mucus is possibly a defense mechanism initiated by the toxins. The circulation of blood in the gills, as well as the flow of water over the gill epithelium can be interfered with by an external film of mucus that coats and clogs the gills immobilizing the gill filaments (Doudoroff, 1957). The liberation of mucus from the gill epithelium increases the diffusion distance between blood and water, therefore affecting the capacity of the gills to transfer gases (Randall, 1970). This in turn would cause the organism to become hypoxic. Support for the occurrence of hypoxia in fishes in the present study was the low blood pO_2 and low % O_2 saturation of hemoglobin.

The increased muscular activity (violent escape responses) of the bluegills exposed to homogenates of L. carteri is believed to be a consequence of hypoxia. Other workers have observed similar reactions of fishes to hypoxia. Marvin and Heath (1968) and Spitzer et al. (1969) found that hypoxic bluegills had an increased rate of opercular movement. Frequent surfacing of the carp, Cyprinus carpio, was observed by Itazawa (1957) when exposed to a low oxygen environment.

The energy required by the bluegill's respiratory apparatus makes up a sizeable portion of the total energy expenditure (Marvin and Heath, 1968). The cost of ventilating the gills in teleosts is considered to be high and

may be as much as 30% of the total oxygen uptake at rest (Randall, 1970). Thus, an increase in respiratory activity of fishes exposed to toxins of L. carteri would increase the demand for oxygen and decrease the amount of oxygen available for other activities, i.e. violent swimming and leaping. It is therefore proposed that there was a partial switch to anaerobic metabolism resulting in a rise in the level of blood lactate. A number of studies show that both hypoxia and exercise cause rapid and significant increases in blood lactate in fishes, including the bluegill (Black, 1955; Heath and Pritchard, 1962; Holeyton and Randall, 1967). Although blood lactic acid was not measured in the present study, indirect evidence for its increase in bluegills exposed to toxins of L. carteri was the occurrence of low pH and HCO_3^- values and high pCO_2 values. The release of muscle lactate into the blood increases the $[\text{H}^+]$ (Satchell, 1971). Hypoxia and exercise in carp increase blood lactic acid, lower blood pH and decrease the HCO_3^- (Satchell, 1971). Holeyton and Randall (1967) found a lowered pH and an increased pCO_2 in Salmo gairdneri (rainbow trout) due to an increase in lactate under hypoxic conditions. The explanation for the lower pH and HCO_3^- and higher pCO_2 due to lactic acid buildup can be obtained by examination of the equation for the elimination of CO_2 from the red blood cell (Randall, 1970):



With an increase in the level of lactic acid, there is an increase in $[H^+]$. Therefore, more H^+ ions are available for combination with HCO_3^- to form H_2CO_3 . This leads to increased levels of CO_2 by the dissociation of H_2CO_3 . The clogging of the gills of fishes exposed to homogenates of L. carteri with mucus would retard the elimination of CO_2 resulting in an increase in pCO_2 and H_2CO_3 . Therefore, an imbalance of the two components, H_2CO_3 and HCO_3^- is found in the buffering system $HCO_3^- + H^+ \rightleftharpoons H_2CO_3$ which is responsible for approximately 70% of the blood buffering capacity in fishes (Albers, 1970). The relationship of HCO_3^- , H_2CO_3 and pCO_2 in the determination of pH is seen in the following Henderson-Hasselbalch equations:

$$1) \quad pH = pK_a + \log_{10} \frac{HCO_3^-}{\alpha pCO_2}$$

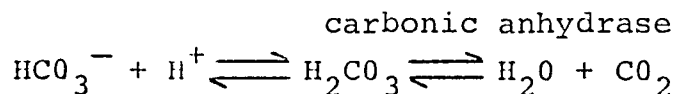
$$2) \quad pH = pK_a + \log_{10} \frac{HCO_3^-}{H_2CO_3}$$

where α is the proportionality constant 0.03 (Gambino, 1963).

In teleost fish, a decrease in pH or an increase in pCO_2 of the blood causes not only a reduction in the affinity of hemoglobin for oxygen (Bohr effect) but also a reduction in the oxygen carrying capacity of the blood (Root effect) (Black, 1940; Ferguson and Black, 1941; Black et al., 1954; Randall, 1970) (Fig. 2). An increase in the concentration of H^+ causes configurational changes in the hemoglobin molecule that inhibit the binding of oxygen

(Satchell, 1971). An increase in $p\text{CO}_2$ not only decreases the affinity of the hemoglobin for oxygen, but renders a portion of it incapable of binding with oxygen at any pressure (Black et al., 1954). Thus, the low pH and high $p\text{CO}_2$ found in blood of bluegills exposed to toxins of L. carteri impair the ability of the fish to withstand hypoxia resulting from the accumulation of mucus on the gill surfaces. It is, therefore, proposed that death in these fishes is due to inadequate blood oxygenation.

Carbonic anhydrase is an enzyme that has been isolated in fish red blood cells and gill epithelium (Maren, 1967). The relationship of carbonic anhydrase with the process of CO_2 elimination from the red blood cell is seen in the following equation (Randall, 1970):



The increased carbonic anhydrase activity found in the blood of the fishes exposed to toxins of L. carteri may be due to the increased number of H^+ ions available for combining with HCO_3^- . The increase in production of the substrate H_2CO_3 would, therefore, produce more CO_2 under the catalytic action of carbonic anhydrase.

Increased carbonic anhydrase activity may also be a result of an increase in the hematocrit of the blood of the experimental fish. An hematocrit increase has been reported for fishes in response to exercise and to reduced oxygen availability. Possible mechanisms responsible for

the relative increase in numbers of red blood cells include splenic contraction, erythrocyte swelling and plasma volume reduction as a result of the movement of water into the tissues from the blood (Kirk, 1974). As carbonic anhydrase is found within the red blood cells only, an increase in the numbers of cells in a unit volume of blood would increase enzyme activity in vitro.

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TABLE 1. Comparison of standard lengths of control and experimental Lepomis macrochirus.

Run	Control (cm)	Experimental (cm)
1	18.2 18.0	18.1 18.4
2	18.1 23.0	17.9 17.9
3	19.4 17.6	17.7 16.4
4	16.9 16.3	15.0 14.2
5	15.0 15.0	14.5 15.2
6	15.1 13.9	15.1 13.9
7	13.6 18.0	17.2 18.1
8	18.2 15.3	16.4 17.8
9	19.5 20.5	19.0 19.1
10	18.5 18.0	18.0 17.5
\bar{X}	17.4	16.9
S.D.	2.3	1.6

TABLE 2. Analysis of variance summary table for standard lengths of control and experimental Lepomis macrochirus.

Source of variance	df	SS	MS	F
between groups	1	2.87	2.87	.703
Error	38	154.61	4.07	
Total	39	157.48		

$$F_{.05}(1,38) = 4.15$$

TABLE 3. The effects of homogenates of Lophopodella carteri on the behavior of Lepomis macrochirus.

Time (min.)	Control	Experimental
start	resting, alert, normal opercular movement	resting, alert, normal opercular movement
15	same as above	widely gaping opercles, appearance of mucus secreted in medium from gill area
30	same as above	infrequent surfacing, snapping movements of mouth, large amounts of mucus secreted in medium from gill region
45	same as above	increased rate of widely gaping opercles, frequent surfacing and breaking of surface with mouth, mucus around gills and in medium
60	same as above	same as above
75	same as above	listing of body from side to side, apparent decrease in rate and amplitude of opercular movement
90	same as above	frequent moments of violent swimming and leaping, large quantities of mucus around opercles
105	same as above	decrease rate of opercular movement, body listing, sometimes completely overturn, mucus still present
120	appeared normal, reacts to stimuli, no evidence of excess mucus	cessation of opercular movement, no response to stimuli, i.e. touch, large quantities of mucus covering the gills and body upon examination

TABLE 4. The effects of homogenates of *Lophopodella carteri* on blood gas properties of *Lepomis macrochirus*

Run	pH		pCO ₂ (mmHg)		HCO ₃ ⁻ mEq/l		pO ₂ (mmHg)		% O sat.	
	Con.	Exp.	Con.	Exp.	Con.	Exp.	Con.	Exp.	Con.	Exp.
1	7.51	7.23	13.75	15.95	8.82	5.36	75.65	12.28	81.0	18.0
	7.43	7.36	10.01	12.10	5.34	5.49	72.80	22.29	79.0	35.0
2	7.42	7.43	14.85	11.00	7.74	5.86	25.90	24.57	49.0	39.0
	7.48	7.52	8.80	11.55	5.26	7.58	27.77	19.11	52.0	29.0
3	7.41	7.29	10.56	11.00	5.38	4.25	34.58	24.57	57.0	39.0
	7.43	7.13	9.90	12.10	5.28	3.23	38.67	16.15	60.0	22.0
4	7.51	7.26	9.68	9.35	6.21	3.37	31.94	29.48	55.0	45.0
	7.73	7.43	8.80	9.62	9.36	5.13	27.39	24.11	52.0	37.0
5	7.26	7.09	9.02	10.23	3.25	2.49	55.15	30.07	68.0	46.0
	7.29	6.93	5.55	6.32	2.14	1.07	59.19	48.09	70.0	60.0
6	7.27	7.10	6.10	7.20	2.25	1.79	43.18	44.36	62.0	57.0
	7.55	6.97	4.61	7.92	3.24	1.46	76.16	45.45	82.0	58.0
7	7.81	7.13	4.01	11.11	5.13	2.97	82.85	33.85	85.0	51.0
	7.34	7.06	9.85	11.33	4.27	2.58	21.61	29.57	46.0	45.0
8	7.62	7.11	7.86	10.89	6.49	2.78	40.49	31.98	61.0	50.0
	7.47	6.96	8.69	11.93	5.08	2.15	33.26	50.73	56.0	61.0
9	7.54	7.03	8.36	12.48	5.74	2.65	25.02	46.59	48.0	59.0
	7.66	7.26	7.15	7.86	6.47	2.83	17.74	23.20	34.0	36.0
10	7.61	7.10	7.48	7.70	6.04	1.92	35.26	38.90	58.0	55.0
	7.31	6.85	7.48	11.44	3.03	1.60	48.68	46.63	65.0	59.0
\bar{x}	7.48	7.16	8.62	10.45	5.32	3.32	43.66	32.09	61.0	45.0
S. D.	.15	.18	2.65	2.25	1.96	1.73	20.04	11.67	13.38	12.93

TABLE 5. Analysis of variance summary table of the effects of homogenates of Lophopodella carteri on pH, pCO_2 and calculated CO_2 in the blood of Lepomis macrochirus.

pH				
Source	df	SS	MS	F
between groups	1	1.03	1.03	36.60*
error	38	1.06	.03	
total	39	2.09		

pCO_2				
Source	df	SS	MS	F
between groups	1	33.43	33.43	5.52*
error	38	230.28	6.06	
total	39	263.71		

Calculated CO_2				
Source	df	SS	MS	F
between groups	1	39.92	39.92	11.60*
error	38	130.31	3.43	
total	39	170.23		

*Significant at the .05 level

TABLE 6. The effects of homogenates of Lophopodella carteri on the ratio of $[\text{HCO}_3^-]$ to $[\text{H}_2\text{CO}_3]$ in the blood of Lepomis macrochirus.

	Mean $[\text{H}_2\text{CO}_3]$	Mean $[\text{HCO}_3^-]$	Mean $\frac{[\text{HCO}_3^-]}{[\text{H}_2\text{CO}_3]}$	Ratio
Control	.26	5.32	$\frac{5.32}{.26}$	20:1
Experi- mental	.31	3.32	$\frac{3.32}{.31}$	11:1

TABLE 7. Analysis of variance summary table for the effects of homogenates of Lophopodella carteri on pO_2 and % O_2 saturation in the blood of Lepomis macrochirus.

pO_2				
Source	df	SS	MS	F
between groups	1	1337.61	1337.61	4.97*
error	38	10222.57	269.01	
total	39	11560.18		

% O_2 saturation				
Source	df	SS	MS	F
between groups	1	2544.02	2544.02	14.68*
error	38	6582.95	173.23	
total	39	9126.97		

*Significant at the .05 level

TABLE 8. The effects of homogenates of Lophopodella carteri on carbonic anhydrase in blood of Lepomis macrochirus.

TRIAL	Control (ul CO ₂ /ml/hr) (wet gas)	Experimental (ul CO ₂ /ml/hr) (wet gas)
1	160 170	160 210
2	230 230	315 180
3	160 145	170 160
4	160 120	60 160
5	60 20	160 150
6	60 80	300 160
7	100 130	120 210
8	240 200	160 200
9	160 120	110 365
10	160 110	140 250
\bar{X}	140	187
S. D.	59	72

TABLE 9. Analysis of variance summary table for the effects of homogenates of Lophopodella carteri on carbonic anhydrase activity in the blood of Lepomis macrochirus.

Source of variance	df	SS	MS	F
between groups	1	21390.6	21390.6	4.84*
Error	38	167783.7	4415.4	
Total	39	189174.3		

*Statistically significant

$$F_{.05}(1,38) = 4.15$$

Figure 1. Procedure for exposure of fish heart

- a. Incision from anus through pectoral girdle
- b. Retraction of tissue exposing heart
- c. Blood removed from heart



a



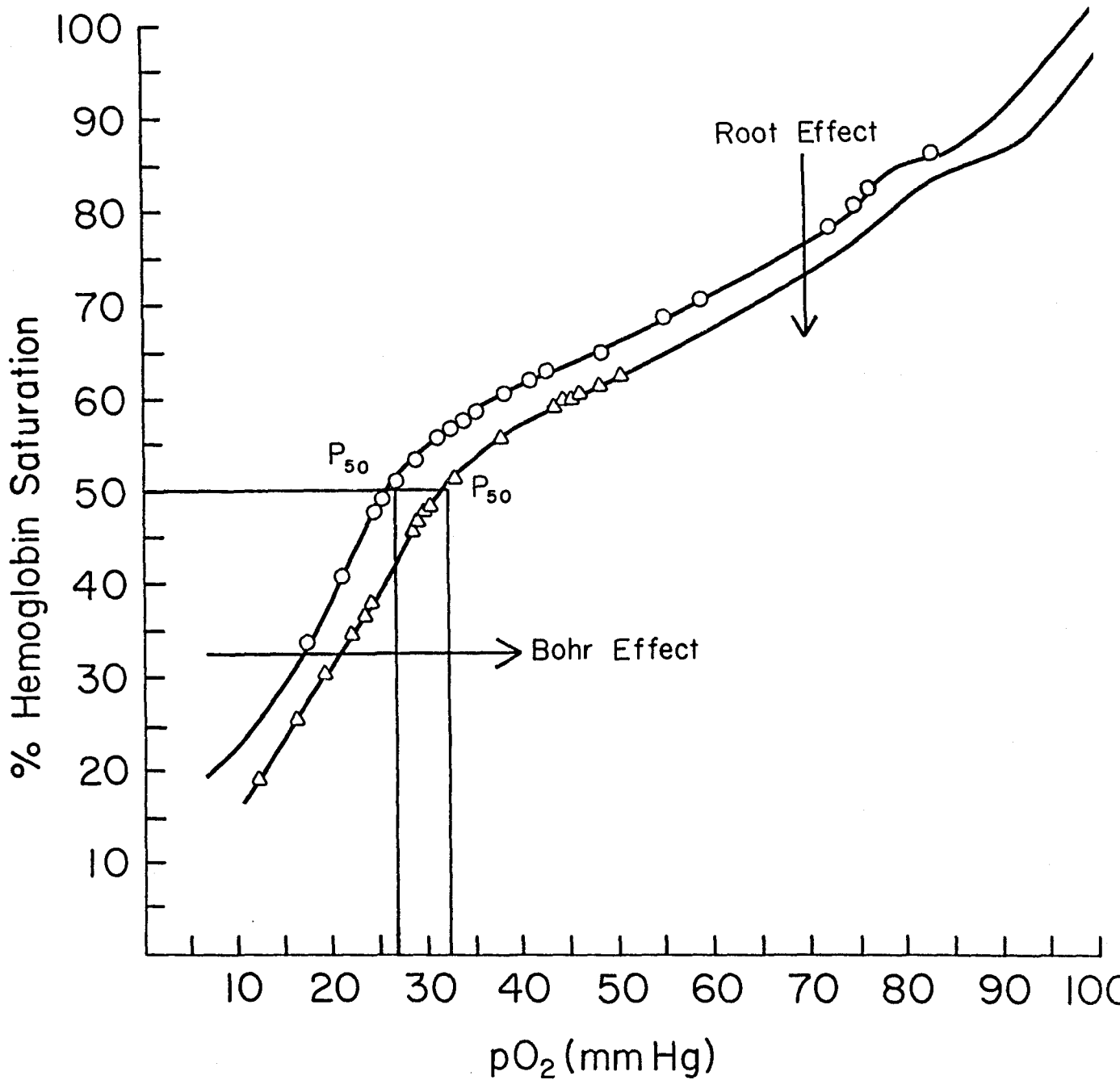
b



c

Figure 2. The effects of homogenates of Lophopodella carteri on the oxygen dissociation curve of Lepomis macrochirus.

○ Control
△ Experimental



VITA

Robert Allen May was born on March 17, 1949, in El Paso, Texas. He moved to Richmond, Virginia in January, 1950 where he received his secondary education at Collegiate School. Following graduation in June, 1968, he entered Hampden-Sydney College where he majored in psychology and biology. He was granted a B. S. degree in May, 1972. In September, 1972 he began his graduate studies at the Graduate School of the University of Richmond in Biology. While there he was elected to Beta Beta Beta Honorary Biological Fraternity. Completion of the requirements for the Master of Science degree was attained in May, 1974. Future plans at this time are indefinite.