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PRELIMINARY STUDIES ON THE EFFECT OF FATIGUE ON THERMAL TOLERANCE OF RAINBOW TROUT, *Salmo gairdneri*

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INTRODUCTION

In the last thirty years a large amount of research effort has been extended in behalf of thermal tolerances of fishes. In a recent publication Coutant (1972) lists thermal tolerance data for more than sixty species. The upper and lower temperature limits for these fishes were derived typically in studies where temperature was the only experimental parameter. That is, the fish were well fed, acclimated to a controlled temperature and external stimuli were minimized. The experimental fish were usually transferred from an acclimation tank to the test tank which contained water at a temperature above or below the acclimation temperature. The thermal tolerance of the species was then determined by the time required for loss of equilibrium or death to occur. The information gathered in this manner is certainly indicative of the thermal tolerance of a species but its application to the natural environment must be conducted with great care.

Today the ecologist is being required to make predictions on the impact of thermal discharges into natural waters on fisheries. Assuming that the ecologist can determine what temperatures are experienced by the exposed organisms, the ecologist must rely upon data typified by originating from laboratory studies like those just discussed. Since,in general, this information was generated under conditions where all parameters save temperature were held at optimal conditions one might question its strict applicability to attempts at quantitative predictions of impact. Fish in their natural state are, of course placed under a number of simultaneous stresses. The waters in which they are swimming may carry pollutants, be deficient in oxygen, supersaturated

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with atmospheric gases, or any number of factors not conducive to the health of the fish population. The combined effect of any one of these factors and a thermal load forces one to be hesitant to strictly apply the available thermal tolerance data.

One of the most obvious differences between laboratory studies and fish in their natural state is that the latter is normally swimming. This is especially true for migrating species, eg. salmonids, which may be in a state of fatigue when they encounter a thermal discharge. The effect of fatigue on the ability of fish to cope with a thermal shock has not been investigated. The combined effect could alter the response of fish to elevated temperatures as they might encounter moving through rivers receiving thermal effluents.

The purpose of this paper is to report on the preliminary investigations designed to provide a basis for defining the combined effects of fatigue and thermal stress on rainbow trout, Salmo gairdneri.

MATERIALS AND METHODS

The fish being used throughout this study is rainbow trout, Salmo gairdneri. The fish were obtained from a commercial hatchery, Troutlodge Springs, Soap Lake, Washington and were maintained in the laboratory for 9 weeks prior to the commencement of experiments. Two lots of fish were used, one obtained Feb. 8, 1972 and one on June 7, 1972. The body size of the fish used ranged from 85-300 gms, the mean weight being 176g (Ave. for 300 fish). The fish were fed dry fish pellets manufactured by Moore-Clark "New Age Fish Pellets", Salt Lake City, Utah. Fish were not fed for 16 hours prior to experimental employment. In all lactic acid and glucose measurements the fish were used only once.

Blood samples for lactic acid and glucose measurements were withdrawn from the fish by syringe placed in the dorsal aorta. A volume of 0.40 ml was withdrawn for both analyses. The average time between removal of fish from water to blood sampling was 0.8 minutes. The lactic acid concentrations was analyzed by Sigma Chemical procedure 826 - UV an adaptation of the Marbach and Weil (1967) enzymatic method. This method depends upon the following reaction:

$$\text{Pyruvic acid} + \text{p - DPNH} \rightleftharpoons \text{L(+) Acid} + \beta\text{ - DPN}$$

which is catalyzed by lactic dehydrogenase and can be used for measurements of both lactic acid and pyruvic acid. In the present application the reaction is driven to the left in the presence of excess β-DPN. The pyruvic acid must be removed by complexing so that the reaction may be driven to completion. The amount of β-DPNH is measured spectro photometrically on a Beckman DU at 340 μ.

Glucose measurements were performed utilizing a Beckman Glucose Analyzer. This instrument measures the rate of oxygen consumption in the reaction catalyzed by glucose oxydase:

$$\text{Glucose} + O_2 \xrightleftharpoons[H_2O]{} \text{Gluconic Acid} + H_2O$$

In this reaction the rate of oxygen consumption is directly proportional to the glucose concentration. The maximum rate observed is therefore a direct measure of glucose in the sample.

In order to facilitate the removal of blood from the fish it is necessary to immobilize the fish by anesthesia. There are a number of methods that are employed for this purpose. It is known that blood levels of both glucose and lactic acid demonstrate a facile response to various stimuli (Caillouet, 1964). A method of immobilization was sought, therefore, that would not introduce error into blood measurements. Several standard fish anesthetics were applied as well as electrical shock and cold shock. The chemicals used were MS 222, Methyl pentynol, and benzocaine. Cold shock was induced by placing the fish into a slurry of ice and ethanol. Five and six fish were used in this study. Fish were acclimated to 12°C and tested at that temperature.

During the investigations of the various means for immobilization it was noted that a resting level of the two blood constituents was very difficult to obtain. Each fish removed from a stock tank would yield slightly elevated measurements over the previous specimen. A method was needed whereby the capture of a fish from the stock tank would not disturb the remaining fish. The method devised appears in figure 1.

A trap door in a connecting tube between two holding tanks provides the means for removing one or two fish from the stock supply, quickly and with minimum disturbance to the fish remaining. The fish are stimulated to swim through the connecting tube by the sight of fish in the adjoining tank. Food pellets dropped through the vent pipe to the trap door also attract the fish. They pass freely through the tube and frequently hover over the trap door. The tube is made of clear plastic to enable observation at a distance. When a fish is in the proper position the trap door is released.

Flap valves partially close both ends of the tube when the trap door opens. The valves restrict the flow of water from the tanks preventing a surge that could excite the remaining fish. Closing the trap door causes water to again fill the tube above the trapping zone, air escapes through a vent tube, and upon equilibration of the water pressure on both sides of the flap valves they return to open position.

The water supply enters through the wall at the top of each tank. Water flows down through each tank into the center of the connecting tube and out through slots in the wall to an overflow standpipe assembly and to waste.

Before attempting to study the combined effects of temperature and exercise on fish a series of experiments were conducted to delineate the response of blood lactic acid and glucose to temperature alone. In these experiments fish were maintained at the acclimation temperature of 12°C for at least four weeks prior to the initiation of the study. For each study fish were removed from the acclimation tank by the method discussed above and placed in an exposure tank at 27°C for three minutes. At the end of this time they were returned to the acclimation temperature. Fish were removed from the acclimation temperature at five minute intervals and blood samples taken for analysis. At each of the thirty-two time intervals four to thirteen fish were taken for blood measurements. The blood sample was then prepared for glucose and lactic acid measurements.

RESULTS

The results of the five methods of immobilization are presented in Figure 1 and Table 1. In all five cases the fish were assumed to be in a rested state, i.e.

swimming freely and undisturbed by external stimuli. Thus when they were removed from the tunnel and anesthetized they were in a state approximating routine metabolism (Job, 1955). It is assumed therefore that the immobilizing method that consistently revealed the lowest measurements for glucose and lactic acid was the method which revealed the most accurate information about the routine levels of each blood constituent. The figure and table indicate that the cold shock method consistently yielded glucose measurements of 69.0 mg % and lactic acid measurements of 7.6 mg %. The lowest for both constituents. These also serve as a baseline measurement for thermal shock study.

As has been indicated above the response of blood levels of both glucose and lactic acid is very rapid following stimulus. Since it is the ultimate goal of this study to determine the effect of combined stresses of elevated water temperatures and exercise (swimming induced fatigue) it was decided that the effect of thermal shock alone should be addressed first. Once this is established the more complicated task of determining the effect of both temperature and exercise can be addressed.

The effect of a thermal shock sustained for three minutes on the blood glucose and lactic acid concentrations has been recorded in table 2 and presented graphically in figure 3. These fish were placed in a 27°C bath directly from an acclimation temperature of 12°C. None of these fish displayed a loss of equilibrium at this exposure. The fish were immediately replaced in the acclimation temperature. Blood samples were taken from the first group of fish five minutes after they first experienced the thermal shock. It can be seen in the data that the groups subsequently sampled reveal a highly fluctuating

level of both glucose and lactic acid. Furthermore these fluctuations occur for a long period of time following the thermal shock. It can also be noted that the rise and fall of both components appear fairly well synchronized at least during the first hour following the shock.

DISCUSSION

The concentrations of lactic acid and glucose in the circulating blood are highly variable and quickly reflect the results of even subtle stimuli upon the fish (Love, 1970). This makes lactic acid and glucose difficult to use as indicators of the organism's metabolic state. However, proper care in handling of both organism and blood samples fluctuations of these blood components can provide useful data.

In the present study the assumption was made that if an accurate measure could be made of the concentration of lactic acid and glucose in a fish in a resting state, this level would serve as a baseline measurement for later comparisons of the combined effects of temperature and fatigue. It was also assumed that the lowest measurements obtained for both lactic and glucose would indicate the method which introduced the least error. A method was sought therefore which would allow the capture and immobilization of a specimen which did not of itself introduce undue elevation of the two blood components.

Several method of capture were attempted before arriving at the design of the apparatus appearing in figure 1. Other methods attempted included dip netting fish from a stock tank and isolation of individual fish in restraint tubes. In the latter method isolated fish were held in tubes for periods of

24 hours prior to blood measurements. Both of these methods resulted in higher average concentrations of blood lactic acid and glucose than did the tank system previously described. A further advantage of the tank system is that it allowed repeated removal of fish from the stock without exciting the remaining stock. Thus the blood measurements remain low through an experiment and the amount of variability in the data minimal.

Once a specimen has been removed from the stock tank, the time before it is immobilized is extremely important to the validity of the measurement. Any undue struggling by the fish results in lactic acid and glucose measurements that do not accurately represent the metabolic state of the specimen. Of the five immobilization methods attempted in this study only one, cold shock, revealed consistently low concentrations of the blood components measured. The three anesthetics tried were MS 222, Benzocaine and methyl pentynol. The MS 222 gave the lowest reading of these with lactic acid at 7.6 mg % and glucose at 69.0 mg %. Even with MS 222, however, there was a short period of struggling before the fish was totally anesthetized and thus emmersion in the ice bath was arrived at the means for immobilization of fish. In the comparison of the different methods cold shock resulted in a blood lactic acid concentration of 7.6 mg % and glucose of 69.0mg %. In the subsequent studies reported here and in the remaining portions of the research the combination of the tank system and cold shock will be used as the capture and immobilization techniques for obtaining blood samples.

The data presented in table 3 provides the details of lactic acid and glucose measurements of blood taken from fish at five minute time intervals following a thermal shock. The fish were acclimated to 12°C and subjected to

27°C for three minutes. The time course of appearance of both lactic acid and glucose shows large fluctuations in concentrations. A review of the literature on the effects of stimuli, predominantly exercise, on the appearance of these components does not reveal similar fluctuations in time. This may in part be due to the frequency of sampling by earlier investigators or be a factor introduced by the thermal shock since the fish in this experiment were not being forced to swim. In fact they were being held in a darkened chamber and swimming movements during the exposure time was minimal.

Previous studies have shown that the major source of the blood lactic acid is the metabolism of muscle glycogen (Black, 1962) Black et al. (1960) could find no evidence to indicate that blood glucose was being utilized by muscle tissue directly in energy production. Further there is evidence that the rate of transfer of lactic acid from muscle to the blood although temperature dependent is remarkably rapid. Despite this rapid transfer the level of muscle lactic acid remains higher than the blood throughout the recovery period (Black, 1962). The evidence from Black's studies indicate therefore that the source of blood lactic acid is the muscle and from this we can conclude that the source of the fluctuations reported here following thermal stress may reflect similar fluctuation in the pattern of release from the muscle. It is intended in the continuing studies to pursue this question. The fluctuations of glucose seen in the present investigation may be closely coupled to those of lactic acid through glucogenesis in the liver. Because the fish used in this experiment had not been fed prior to the tests the only source of blood glucose was the liver. It is hypothesized that the lactic acid released into the blood from the muscles would be transported to the liver where it would be converted back to glucose. It is possible that this glucose could then be released back into the blood for transport to the muscle.

This explanation seems to be supported by the close relationship of fluctuations for both lactic acid and glucose following the thermal shock.

The work conducted in this study thus far is considered preliminary to experiments which will be designed to determine the combined effect of temperature stress and fatigue. It is intended that these studies will reveal whether fish in a fatigued state display a lowered resistance to elevated temperature. Included in this research will also be studies of the basic biochemistry of energy metabolism during muscular activity and exposure to elevated water temperatures.

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Table 1. Comparison of immobilization methods for Rainbow trout. Fish acclimated to 12°C, measurements made at that temperature. Lactic acid and glucose measurements given in mg %.

Method of Anesthesia	Number of Observations	Glucose (Mg%) Geometric Mean	Glucose (Mg%) Range	Lactic Acid (Mg%) Geometric Mean	Lactic Acid (Mg%) Range	Mean wt.
MS 222	5	76.8	73.5-80.0	8.3	5.2-11.4	222
Methyl Pentynol	5	74.4	65.8-82.9	14.1	10.8-17.5	216
Benzocaine	5	71.6	55.1-88.5	9.5	5.8-13.1	220
Cold-Shock	6	69.0	61.8-76.1	7.6	3.45-18.6	306
Electro-Shock	6	79.3	62.1-96.6	10.8	0.28-21.9	214

Table 2. Blood lactic acid and glucose concentrations, mg %, following 3 minute exposure to 27°C. Rainbow trout acclimated to 2.5-4.9°C. Time zero is taken from the point of removal from the acclimation temperature. Mean weight of the fish was 190.7.

Time(min)	N	Glucose (Mg%) Mean	Glucose (Mg%) Range	Lactic Acid (Mg%) Mean	Lactic Acid (Mg%) Range
0	5	89.2	58.8-119.6	6.62	5.27-7.98
5	5	80.6	68.6-92.6	36.0	23.8-48.3
10	5	98.6	76.5-120.7	28.0	22.9-33.2
15	5	111	91.2-130.8	48.4	35.9-60.9
20	5	103	84.3-121.7	32.0	21.1-42.8
25	5	103.8	79.0-128.6	31.0	18.2-43.9
30	5	115.6	102.9-128.3	45.9	32.3-59.4
35	5	120.4	101-139.8	46.4	35.7-57.1
40	5	107.4	96.9-117.9	28.7	21.6-35.8
45	5	118.6	99.8-137.4	22.0	14.4-29.7
50	5	119.2	100.7-137.7	18.1	11.3-25.0
55	5	93.8	81.9-105.7	24.6	0.92-48.3
60	6	111.5	87.8-135.2	37.1	18.3-56.0
65	5	101	84.1-117.9	36.2	2.93-69.5
70	5	101.6	78.8-124.4	15.0	9.60-20.4
75	5	105.4	99.6-111.2	36.6	16.3-56.8
80	5	98.8	89.8-107.8	28.9	10.4-47.4
85	5	136.6	118.8-154.4	38.5	19.8-57.2
90	5	111.8	94.8-128.8	30.4	12.6-48.3
95	5	113.8	98.4-129.2	18.9	-8.50-46.2
100	5	103	92.1-113.9	23.9	-0.99-48.8
105	5	97	74.3-119.7	10.0	-1.73-21.7
110	5	121	88.8-153.2	9.5	-6.19-25.1
115	5	110.2	78.1-142.3	18.0	-5.53-39.0
120	9	103	89.3-116.7	15.7	-0.91-32.4
125	13	102.2	89.8-114.7	33.3	16.9-49.7
130	6	107.5	82.5-132.5	19.8	2.48-39.6
135	12	125.3	106.5-144.0	26.7	16.2-37.2
140	5	126.8	114.8-138.8	9.2	-3.68-22.1
145	5	107.4	85.0-129.8	20.9	11.5-30.3