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Aspects of Stress in Rainbow Trout, *Salmo gairdneri*—Release of Chemical Alarm Signals* [1]

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Changes in the metabolism of rainbow trout caused by stressors of different nature and intensity (electric current, injections, handling, and their combinations) were studied. Under stress caused by electric current, the trout releases metabolites into the water that induce changes in recipient behavior. Urine and feces seem to be the main sources of "stress pheromone," The substances are thermostable, have molecular weight less than 1500, and maintain biological activity for one day.

Key words: Rainbow trout, *Salmo gairdneri*, *Oncorhynchus mykiss*, aspects of stress, chemical alarm signals

Environmental factors influencing fishes may cause changes in metabolism which in turn may indirectly provide information about the environment to other fish. The strength of the effect is determined by the intensity of the factor which influences the fish in a stimulating and stressful way. Changes in the physiological status of fish under stress provide insight into the causal interactions between the environment and the fish. Different intensities of stress suggest different intensities of response of the fish at all levels of its organization and, therefore, different changes in metabolism. The latter relates to changes in external metabolites (EM) which are released by fishes under stress. These EM are different in quantity, proportion, and possibly chemical nature from EM released by intact specimens and can communicate information about danger. During chemoreception, this information is recognized by fish of the same and sometimes by other species. Metabolites released into the environment by fish in normal physiological condition seem to be stressors at increased concentrations (Lebedeva and Golovkina, 1986). Pheromone of alarm and kairomone of predatory fish have been discovered and partially purified; they are chemical signals of danger to quiescent fish (Lebedeva and Chernyakov, 1978; Kasumyan and Lebedeva, 1979; Lebedeva et al., 1982). Alarm pheromones are not known for predatory fishes. But some predatory fish are known to recognize an alarm signal by chemoreception. By chemoreception, cod react to fright (water taken from other cod exposed to stress) by increasing avoidance behavior. Electric current caused the maximum avoidance behavior of recipients (Marusov, 1989).

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[1] The American Fisheries Society classification of rainbow trout has changed *Salmo gairdneri* to *Oncorhynchus mykiss*.

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The goal of this study was to evaluate the stress reactions of rainbow trout and to search for released EM which can be recognized by recipients and cause various physiological reactions. The stressors were electric current, handling, injections, hypoxia, and their combinations. Water taken from aquaria holding stressed fish was also used as a stressor. The criteria of stress were intensity of respiration (frequency of respiratory movements), concentration of glucose in blood, and biochemical composition of mucus.

Materials and Methods

The study was performed in the autumn of 1987 and 1988 and in the spring of 1989 using 150 male and female rainbow trout weighing 210–370 g and measuring 24–29 cm. Eighteen hours before an experiment, a single rainbow trout was placed into a 200-liter aerated aquarium. The fish were exposed to conditions causing varying degrees of fright and accompanying stress. The stress factors of danger of different nature and intensity were electric current, injection, handling, and their combinations. The parameters of electric current were 30 s duration, 100 Hz frequency, 0.8 ms pulse width, 40 V/m field strength. This stimulus immobilized the fish for 5 s. To determine the presence of metabolites released by stressed trout, 3 min after imposing the electric current, 1 to 5 specimens were removed from the aquarium and then intact fish were introduced for two hours. Some control experiments were performed where intact fish were (1) placed in water through which electric current had been passed in the absence of fish; (2) placed into water where a group of fish were previously kept without stimulation; (3) placed in an aquarium with fresh water. To determine the properties of metabolites released into water in which fish had been subjected to electric current, the water was kept at room temperature for one day, heated to 100 °C for one hour, and boiled for 10 minutes. Changes in metabolism due to the frightening stimulus were evaluated by physiological and biochemical criteria of stress. The physiological criterion was frequency of respiratory movements (FRM, per min); it was measured over 3 min (results are expressed in percent). The biochemical criterion was the concentration of glucose (mmole/liter) in the blood of the trout. Glucose concentration was determined by two methods: a rapid method with reagents while the fish was immobilized on a solid support of multilayer film (Ames), and an electrochemical method using an EKSAN-G instrument (Vosilene, 1988). Values of glucose concentration obtained by the rapid method were slightly higher than those obtained by the electrochemical method. If glucose concentration exceeds the range of values 2.5–7.5 mmoles/liter, there is incomplete agreement of the results obtained by the two methods; however, the tendency for change of glucose concentration after stimulation remains. Therefore the rapid method for determination of glucose in blood, which is widely used in medicine and requires no special instruments, can be used for trout under field conditions as shown for carp (Lebedeva and Golovkina, 1990). Changes in the physiological and biochemical parameters of metabolism were determined two hours after the stimulation.

In the spring experiments, metabolites were obtained from fish with thin elastic rubber bags attached either over the posterior part (up to the gills) or over the anterior part to the dorsal fin) of the body after exposure to electric current. After the experiment, the contents of the bag (designated as EM) were poured into a glass vessel, the bag rinsed 3–4 times with water, and all this water was added to the aquarium for testing fish. A water-filled bag was exposed to the electric current, and water from it was used as a control stimulant to determine the possible effect on trout of the bag odor itself.

To separate EM, gel chromatography was performed with Sephadex G-15 under conditions used for fish pheromones and kairomones (Lebedeva et al., 1982). Both the low-molecular-weight fraction (EM_{II}) and the high-molecular-weight fraction (EM_{I}) were collected for further testing.

Mucus for testing was collected with a blunt histological spatula and then mixed in 100 ml of water and presented to fish. Attempts to alleviate stress and its possible negative consequences were made using delta-sleep peptide. This peptide at 220 μg/kg fish weight was injected into the abdomen 1 hour before the exposure to the stressor (Lebedeva et al., 1988).

External mucus potassium and sodium concentrations were measured using an IL-943 semiautomatic flame photometer. The procedures for removing and preparing mucus for analysis were described by Lebedeva et al. (1988). The concentration of electrolytes is expressed in milli-equivalents per gram of dried mucus.

To determine formed elements (erythrocytes, lymphocytes, and others) in external mucus, smears were made on mounts, dried and fixed in absolute alcohol for 15 min. They were then stained by the Romanovskiy-Giemsa method of with azure and eosin (Volkova and Yeletskiy, 1982).

Results

The values of every biochemical parameter varied under normal conditions. In 1988, FRM ranged from 58 ± 3.0 to 75 ± 5.6 in control specimens. For comparison and to determine the relationship of FRM changes to stimuli for specimens with different initial values of the parameter, the data are given in percent (Table 1). Shortly after imposition of the stressors, an intensification and change in the respiration pattern was observed. For example, during the first minute after imposition of the electric current, the respiratory rate was reduced and respiration was deeper and irregular; during the next 2–3 min, the respiratory rate was faster, and during the next 2–3 min it became normal. Two hours after the imposition of some stressors, FRM was still increased. Water taken from fish exposed to electric current caused the largest increase in respiratory rate. Respiration did not normalize after the imposition of some stressors in combination (for example, injection of saline and electric current). Intensification of a stressor by increasing the time the fish donor was kept in the water and decreasing the volume of the water where the fish was exposed to stress (to increase the concentration of released metabolites), and also increasing the time of exposure of fish recipients caused a small change of respiratory intensity due to the effects of EM induced by electric current or hypoxia (Table 2). In different seasons of the year, the intensity of the reaction of fish differed; this may be due to differing geometeorlogical conditions associated with the season which affected the fish differently.

Glucose concentration in the blood of control fish averaged 2.9 ± 0.14 mmoles/liter. Hyperglycemia was found in all trout exposed to any of the stressors. After stimulation with electric current, glucose concentration averaged 4.7 ± 0.33 mmoles/liter. Injections and handling caused only mild hyperglycemia (see Table 1). The level of hyperglycemia did not further increase in recipients when the number of fish donors was increased. Also, the hyperglycemia did not depend on the level of stress of the fish donors. The effect of two factors of danger, that is, stimula-

Table 1

Comparison of Changes in Respiration Frequency and Glucose Concentration in Blood of Rainbow Trout Exposed to Different Stressors Measured by Two Methods (autumn of 1968)

Acting factor	Respiration frequency after 2 hours (%)	Glucose after 2 hours			
		EKSAN-G apparatus		Multisticks	
		mmole/liter	%	mmole/liter	%
Control	100	2.9±0.14	100	3.2±0.35	100
Water exposed to electric current in the absence of fish	101	3.0±0.15	103	3.4±0.65	106
Electric current	101	4.7±0.33	162	3.5±0.34	165
Peptide injection	108	3.5±0.27	121	4.8±0.61	150
Saline injection	98	3.5±0.15	121	4.5±0.30	141
Handling	102	3.2±0.20	180	4.0±0.25	180
Water from fish exposed to electric current	131	3.9±0.24	134	4.7±0.10	148
Peptide injection + electric current	112	5.5±0.67	193	6.2±0.52	194
Saline injection + electric current	113	6.6±0.63	227	6.9±0.46	216
Peptide injection + water from fish exposed to electric current	106	4.4±0.27	159	4.9±0.38	153
Saline injection + water after fish exposed to electric current	117	3.9±0.39	135	5.1±0.45	159
Handling + electric current	110	5.5±0.20	220	6.0±0.25	220

tion by electric current 1 hour after injection of saline or peptide, caused much greater hyperglycemia than the electric current alone. Two hours after imposing these two factors, FRM did not normalize. The strong stressors probably caused a change in fish metabolites revealed by the increase of both FRM and glucose concentration in blood; thus, the character and strength of the resulting changes correlate with the intensity of the stimulator and the progress of the stress response in trout. The observed changes in metabolism suggest the composition or relationship of metabolites released changes during stress. In control experiments, water taken from control specimens

Table 2

Changes of Physiological and Biochemical Parameters of Rainbow Trout during Stress (spring of 1990)

Stimulator*	Duration of treatment, min	Respiration, %		Glucose in blood after 120 min, %	Mucus											
		5 min	60 min		Sodium, meq/g	%	Potassium, meq/g	%	Potassium/sodium, %		Ketones, mmole/liter	Protein, g/liter	Hemoglobin, μg/liter	Density, g/cm³	pH	
1. Electric current	10	131	128	210	51.5	75	42.2	81	0.88	108	0.50	0.17	258	1.008	7.2	Control
	60	136	106	200	60.6	88	57.2	110	1.05	130	0.75	0.10	155	1.010	7.2	Water from current
1a. Water	10	124	110	120	38.8	56	34.0	65	0.79	97	0.70	0.23	258	1.010	7.0	With elasti
	60	152	123	135	83.0	121	62.8	120	0.82	101	0.75	0.55	232	1.010	8.0	Water from current +
2. Handling	10	130	125	156	44.1	64	28.8	55	0.65	80	0.70	0.53	155	1.007	7.5	EMp
2a. Water	10	129	119	132	43.8	64	42.6	80	1.00	123	<0.50	0.23	362	1.008	7.3	EMp from current
3. Hypoxia	10	133	123	197	70.0	102	33.2	63	0.49	60	<0.50	0.36	310	1.010	7.2	EMa from current
	60	152	140	207	82.8	121	57.2	109	0.69	85	0.50	0.20	310	1.007	6.6	Insoluble fi
3a. Water	10	134	124	155	54.0	79	41.2	78	0.77	95	« 0.50	0.28	263	1.008	7.3	Fraction I c
	60	135	117	150	27.5	40	21.3	40	0.77	95	0.70	0.65	155	1.007	6.0	Fraction II
Control		107	97	100	68.6	100	52.5	100	0.81	100	0.50	0.33	155	1.010	6.9	Content of exposed
																Control (w shed")
																Control (ae
																Water from current: t
																Incubation for 1 day

* (1)–(3) Treatment; (1a)–(3a) water from treated fish.

and water where electric current had been imposed in the absence of fish did not cause hypoglycemia or increase in FRM.

The biochemical composition of epithelial mucus from control trout was studied; the concentration of protein, ketones, hemoglobin, the density, and pH were determined (see Table 2). During stress, values of these parameters vary, but there was no correlation between the changes and the level of stress imposed for most of the studied parameters. The presence of hemoglobin in trout mucus was found by a histological method. The number of blood cells in mucus is estimated as percent of the total number of cells. During stress, the number of erythrocytes in mucus does not increase (only individual erythrocytes occur), but the number of blood cells increases threefold (from 1.5 to 4.5 percent) in comparison with control fish. It should be noted that during stress the quantity of skin mucus decreases.

Sodium and potassium contents and their ratio in mucus are informative biochemical parameters of physiological status which correlate with the changes in glucose concentration in blood. Potassium and sodium concentrations vary considerably in control fish, but their ratio is rather constant, that is, 0.8–0.9 (see Table 2). The imposition of some stressors considerably decreased the concentration and ratio of the electrolytes; the imposition of EM from fish exposed to the stressors does not decrease the ratio and also does not reveal clear tendency in change of the electrolyte relationship in recipients.

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Table 3

Change in Respiratory Frequency and Glucose Concentration in Blood of Rainbow Trout during Stress (spring of 1989)

Stimulus	Respiratory frequency, %		Glucose	
	5 min	2 hours	mmole/ liter	%
Control	Not det.	100	3.00	100
Water from fish exposed to electric current	Not det.	78	4.50	150
With elastic bag attached	102	104	3.00	100
Water from fish exposed to electric current + elastic bag	145	104	7.25	241
EMp	Not det.	102	5.00	167
EMp from fish exposed to electric current	122	99	5.75	192
EMa from fish exposed to electric current	113	105	4.75	158
Insoluble fraction of EMp	108	98	4.38	146
Fraction I of EMp	108	95	4.83	161
Fraction II of EMp	97	105	6.00	200
Content of control bags with water exposed to electric current	106	88	2.70	92
Control (water of "native water-shed")	109	90	2.50	93
Control (aerated water)	102	87	2.50	77
Water from fish exposed to electric current: boiled	107	105	4.70	163
Incubation at room temperature for 1 day	123	143	2.85	98

The composition of mucus after imposition of electric current was studied as a possible source of EM stimulating fish. Mucus of fish exposed to electric current did not increase the FRM or glucose concentration in blood in 1 of 10 experiments. Perhaps metabolites are rapidly washed from mucus to the external environment. But there is another, more probable, explanation of the results: The main source of chemical alarm signals is from excrement and urine, as secondary products of metabolism released with them. To test this assumption, EM were collected from the anterior (EMa) and posterior (EMp) parts of the body and used in experiments. The EM caused small changes in the studied parameters. However, EM from fish exposed to the combined effects

of electric current with the elastic bag attached, and also EMp from fish exposed to the same effects caused the maximum increase in glucose concentration, up to 200 percent. Since EMp from fish donors caused greater changes in the physiological and biochemical parameters, the concentrated metabolites were exposed to chromatography. Then high-molecular-weight (molecular weight more than 1500) and low-molecular-weight (molecular weight less than 1500) fractions were tested with recipient fish (Table 3). EMp from fish donors caused the maximum hyperglycemic effect in recipient fish. Two hours after exposure to the low-molecular-weight EM fraction, FRM was normal. A study of the thermal stability of the active metabolites indicated that they are not proteins. After heating, water taken from fish exposed to electric current (containing active metabolites) did not lose its specific activity for recipients. However, in recipient fish, water taken from fish exposed to electric current and incubated at room temperature for 24 hours increased FRM, but did not cause hyperglycemia. The high-molecular-weight and insoluble fractions of EM caused an increase in FRM in recipients which normalized in two hours. The recipient fish also became hyperglycemic.

The bioregulatory delta-sleep peptide which has a sedative effect in mammals was tested in trout; it normalized FRM, but hyperglycemia remained. A study of stress in silver carp using the delta-sleep peptide gave similar results (Lebedeva et al., 1988).

Discussion

The various levels of progression of stress can be evaluated by selected parameters. In trout under stress, changes in the depth and rate of respiration and increase in blood glucose concentration have been shown. During the progression of stress, a decrease in quantity of external mucus, the appearance of an increased number of blood cells in mucus, and a change in the concentration of some biochemical components (especially univalent ions) have been found. The concentration of biochemical markers in mucus is species-specific (Lebedeva, 1978). Thus, in carp, higher protein content and lower pH were found for mucus. In comparison with carp, trout have lower concentrations of univalent ions. To evaluate physiological status, at least three parameters should be used to prove the presence of a syndrome.

Considering the results, it should be noted that three parameters showing changes in blood and mucus, and also a change in physiological status can reliably prove the presence of a syndrome, and indicate the extent of its progression as well. EM taken from fishes exposed to hypoxia and handling cause increases in glucose concentration and respiration in recipients, but the ratio of ion concentrations does not change. In fish donors exposed to stress, a significant increase in all three parameters was found.

In this case, the first stage of stress may be occurring with further progression of stress. The "main" parameter for the evaluation of stress is still difficult to distinguish. In stress, the respiratory parameter increases first, then the motor parameter, and finally secretion increases. In fish, the extent of changes in metabolism depends on the intensity of the stimulus, which may be expressed either qualitatively or quantitatively. Moreover, season of the year and individual reactivity of fish affect changes in metabolism under the influence of alarm factors. With changing geophysical conditions, a decrease in the extent of the reaction to the stimulus was found.

During exposure to electric current, trout release into the medium substances which other trout recognize as a danger signal; this "alarm pheromone" gives a signal to recipients about the stress and causes biochemical changes in the recipients. During the first minutes after imposition of a stressor, metabolites released by the fish appear in the medium. It should be noted that neither the level of stress nor the number of stressed fish influence the stress level of the recipients. The hyperglycemia observed in most cases after the imposition of electric current exceeded the hyperglycemia observed after exposure to the alarm pheromone. In some fish, exposure to the electric current caused glucose concentration to increase up to 7–8 mmoles/liter, but after exposure to metabolites, the glucose concentration never exceeded 4.5 mmoles/liter. Biologically important chemical signals may cause increases in glucose concentration within the range of physiological regulation by homeostasis, but concentrations lying beyond this range disturb regulation. The cumulative effect of two stressors showed the importance of interpreting processes occurring in nature when several stimulators affect an organism. Increasing the time of exposure of donor fish to stressors and also increasing the time of exposure of recipient fish to EM from the donor did not reveal significant increases in biological activity.

Chemical alarm signals leave "traces" with informative significance concerning the presence of fish exposed to stress. When trout are exposed to hypoxia and handling, metabolites released into the water cause hyperglycemia and increased FRM but do not change the ratio of univalent ions in mucus. In our opinion, a possible explanation for this phenomenon is that fish release metabolites whose chemical properties or quantities differ in relation to the characteristics of the stressor. Electric current may produce a specific effect on the integument. However, the EM in mucus do not have information about this stressor. In trout, as in some cyprinids (Lebedeva and Golovkina, 1987), mucus changes during stress: its quantity decreases and the concentrations of some biochemical components, especially ion concentration ratios, change. Also, no correlation between hemoglobin in mucus and the level of stress has been shown (in contrast to carp: Lebedeva and Golovkina, 1989) in spite of the fact that an increase in blood cell number in mucus was found during the progression of the syndrome. The high thermostability, the retention of biological activity of the alarm pheromone for up to one day, and its low molecular weight apparently allows trout to "mark" a dangerous area for a long time with a biogenic signal. The significant effect caused by metabolites released by the posterior portion of the body suggests that metabolites in excrements and urine are the main source of alarm pheromone. Also, in trout, substances released into the environment through the skin and with mucus caused some changes in physiological and biochemical parameters.

As mentioned, low-molecular-weight substances in the skin of predatory fishes is species-specific (Lebedeva and Chernyakov, 1978). To make an overall "chemical picture" of danger, trout probably need several substances, but, for knowledge about the stressful status of a fish after the imposition of a stressor, the substances released with the final products of metabolism are probably sufficient.

Conclusion

Under strong stress, rainbow trout released external metabolites with specific biological activity. Recipients recognize these metabolites as a signal of danger, or alarm pheromone. For trout