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The physiological stress response to acute thermal exposure in Black Sea trout (*Salmo trutta labrax* Pallas, 1814)

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Abstract: In this study, we aimed to investigate the acute stress responses of Black Sea trout to acute thermal fluctuations. Black Sea trout (184.22 g mean weight) were transferred from a private trout farm to a research center and restocked in a fiberglass square tank. After acclimation to culture conditions (at a water temperature of 15 °C) for 2 weeks, the fish in the experiment were exposed to 25 °C water temperature for 30 min, and the stress responses of the fish were examined at 1, 3, 6, 12, 24, 36, 48, and 72 h. After acute thermal shock, cortisol and glucose levels increased during the first hour in the experimental group. However, cortisol and glucose levels returned to their normal values after 6 and 36 h, respectively. Lysozyme activity decreased at 3 h and rose to its normal level at 12 h after the experiment. Total protein and serum ion concentrations were also affected and fluctuated due to acute thermal stress. Short-term acute thermal exposure also caused a serum ionic imbalance in the trout, but homeostasis was restored at 72 h.

Key words: Black Sea trout, plasma cortisol, glucose, lysozyme activity, total protein and ion concentrations

1. Introduction

The Black Sea trout (*Salmo trutta labrax* Pallas, 1814) is an endemic trout species inhabiting coastal Black Sea waters and streams that drain into the Black Sea coast of Turkey (1). Currently, the Black Sea trout is potentially important for the Turkish aquaculture industry and has been cultured on a small scale in almost all trout farms operating in the Black Sea region (2). Free-swimming Black Sea trout are known to move to preferred temperatures due to the constant changes in water temperature. Thus, Black Sea trout migrate from the sea to rivers and creeks at the end of spring and from rivers to the sea to preferred temperatures of 20–25 °C after spawning when the sea temperature is low (1,3). The farming of Black Sea trout in cages limits their movements between the sea and rivers. In some years, farmers need to delay the harvesting of fish and stock them in cages until late spring because of low market demand and low prices. Thus, Black Sea trout can be exposed to high water temperatures when the Black Sea's water temperature reaches 20–25 °C in late spring. The water temperature can even surpass 25 °C during the summer season.

Water temperature is more important than other environmental factors because it affects all biochemical and physiological processes in fish (4). All fish species have a species-specific thermal limit and exhibit different behaviors and physiological responses during thermal fluctuations (4,5). Every fish species has its own thermal tolerance limit depending on its thermal acclimation capacity and they may demonstrate different reactions while acclimating to new thermal conditions (6).

Prolonged or acute exposure to nonlethal water temperatures causes physiological stress, adversely affecting fish homeostasis and inducing the secretion of steroids such as cortisol, which is an indicator of physiological stress in teleost fishes (7). Stress responses may include an increase in glucose levels to support increased energy utilization and an elevation of lysozyme, which is a protein involved in the innate immunity in fish (8).

There are some studies on the effect of temperature as a stressor on other salmonid species. Warming studies have reported increased serum glucose and cortisol levels in juvenile Chinook salmon (9) and rainbow trout (10). Similarly, an increase in water temperature caused the elevation of plasma cortisol and lactate levels in sockeye salmon (11). However, the effects of temperature fluctuations differ depending on the species. Thus, there was no elevation of the serum cortisol level in Arctic charr (*Salvelinus alpinus*) (12) subjected to daily water temperature fluctuations in the range of ±5 and ±1 °C.

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Plasma ion concentrations are also indicators of fish homeostasis, and they are generally stable under unstressed conditions (13). An increase in the ion flow induced by stress can cause imbalances and fluctuations in plasma ion concentrations in fish. Changes in plasma ion concentrations may be a result of fish adaptation to new conditions. Fish need high amounts of dissolved oxygen during acute stress so that the water flow through the gills can increase (14).

Physiological stress responses in cultured fish depend on their ability to acclimate to different water temperatures. Therefore, the thermal biology of Black Sea trout in both wild and cultured populations is very important. Although there are numerous studies on the thermal physiology of trout and other fish species, information is lacking on the thermal physiology and stress of Black Sea trout. In this study, cortisol, glucose levels, lysozyme activity, total protein, and plasma ion concentrations were studied to better understand Black Sea trout thermal stress responses following an acute thermal shock.

2. Materials and methods

2.1. Experimental fish

Experimental fish (184.22 ± 10 g mean weight and 23.09 ± 3 cm mean total length) were obtained from a private trout farm in Trabzon, Turkey. The fish were transferred to Recep Tayyip Erdoğan University's Aquaculture Research Unit of the Fisheries Faculty in Rize, Turkey. A total of 120 fish were restocked into square fiberglass tanks (400 L) and left for adaptation for 2 weeks. During the adaptation period, the fish were fed twice daily with commercial trout feed (42% raw protein, 22% raw fat, 12% raw ash, and 4350 kcal kg^{-1} energy). The tank was aerated with a bottom diffuser and supplied with 2 L min^{-1} of spring water (14–15 °C).

2.2. Thermal challenge

Two groups were formed, one serving as a control and the other as an experimental group. Before the experiment, 5 fish were sampled from the fish acclimated to a water temperature of 15 °C for 2 weeks. Following this, 55 experimental fish were transferred to a square thermal challenge tank (400 L) at 25 °C, and the temperature remained constant for 30 min with a recirculating miniature water heater-cooler system (Frigotek, İzmir, Turkey). At the same time, 60 control fish were also netted to equalize the handling stress, and the water supply to their tank was stopped for 30 min. After 30 min, the water temperature in the experimental tank was reduced from 25 °C to 15 °C at a speed of 1 °C min^{-1} by adding spring water. The water temperature was recorded as 14.9 ± 1.1 °C during the experiment, except during the 30 min of acute thermal challenge. Both the experimental and control tanks were aerated. Dissolved oxygen was 7.85 ± 1.35 mg L^{-1}.

2.3. Blood sampling

At 1, 3, 6, 12, 24, 36, 48, and 72 h after the beginning of the experiment, 5 fish per tank (control and experimental) were carefully netted and dried with paper towels before the cutting of the caudal peduncle without being anesthetized. Blood samples (by caudal puncture) were immediately transferred to potassium ethylenediaminetetraacetic acid-coated centrifuge tubes and stored on ice. Blood plasma for analysis of cortisol, lysozyme activity, and glucose and for determination of plasma ion concentrations was obtained by centrifugation of a whole blood sample at 10,000 × *g* for 5 min. The plasma samples were stored at a temperature of −18 °C until further analysis.

2.4. Biochemical measurements

Cortisol levels (μg dL^{-1}) were determined with a gamma-coated radioimmunoassay (RIA) kit (Active Cortisol RIA DSL-2100, Diagnostic System Laboratories, Inc., Webster, TX, USA) according to the manufacturer's instruction using a gamma counter (Genesys Gamma-1 Single Detector RIA Gamma Counter, Laboratory Technologies, Maple Park, IL, USA).

Lysozyme activity (U mL^{-1}) was determined using a *Micrococcus lysodeikticus* (Sigma-Aldrich) lysozyme assay in fish plasma according to Engstad et al. (15) with slight modifications. Bacterial cells (0.2 mg mL^{-1}) in 0.05 M sodium phosphate buffer were used as a substrate. Blood plasma (90 μL) was added to 2910 μL of the bacterial suspension. After 30 s and 4.5 min of mixing at room temperature, the decrease in absorbance was recorded at 540 nm using a UV-Vis spectrophotometer. A decrease in absorbance of 0.001 U min^{-1} was considered a sign of lysozyme activity.

Plasma total protein, glucose, calcium, sodium, chloride, potassium, and phosphate were determined using commercial kits (Cormey Accent 300, Poland) and an autoanalyzer (Mindray-BS 400, China) following the manufacturers' instructions.

2.5. Statistical analysis

All data were analyzed using one-way analysis of variance (ANOVA) with SPSS 16.0. The significance of differences between means was compared by Duncan's multiple range tests. Differences between means were considered significant at the 0.05 probability level.

3. Results

After the thermal challenge, the cortisol level changed from 1.07 ± 0.24 to 9.54 ± 0.74 μg dL^{-1} in the experimental group. The cortisol level increased in the first hour and, after 3 h, stayed at levels between 7.29 and 9.54 μg dL^{-1} for the next 2 days. The highest plasma cortisol level was 30.19 ± 1.17 μg dL^{-1} at the 1-h mark following the challenge in the heat-shocked experimental group, and it decreased to 10.69 ± 2.66 μg dL^{-1} after 6 h. The cortisol level differed

significantly ($P < 0.05$) between the groups in the first 3 h (Figure 1). After 6 h, the cortisol level decreased, and the mean cortisol level was not statistically different between the control and experimental groups.

The plasma lysozyme activity in the stressed group decreased significantly ($P < 0.05$) between 3 and 6 h after the thermal challenge, from an initial 533 ± 27.38 U mL^{-1} to 201.11 ± 72.67 U mL^{-1} at 3 h. The activity level returned to 455.55 ± 35.78 U mL^{-1} within 12 h. The plasma lysozyme activity values in the control group fluctuated between 488.88 ± 18.92 and 558.88 ± 19.61 U mL^{-1}, and the mean values did not differ significantly for all time points (Figure 2).

The plasma glucose levels increased from 12.80 ± 1.2 to 62.00 ± 3.03 mg dL^{-1} in the experimental group and then decreased at 72 h. The mean glucose level fluctuated between 12.80 ± 1.2 and 34.00 ± 5.67 mg dL^{-1} in the control group. The glucose levels in the control and experimental groups were significantly different ($P < 0.05$) at 36 and 72 h (Figure 3).

The detected concentrations of plasma total protein, chloride, calcium, potassium, sodium, and phosphate in both groups are given in the Table. In the experimental group, the total protein level increased from 2.44 ± 0.41 to 3.2 ± 0.29 mg dL^{-1}, and this increase was found to be statistically significant ($P < 0.05$); however, at the following time points, the total protein levels fluctuated between 2.72 ± 0.16 and 3.64 ± 0.25 mg dL^{-1}.

The minimum and maximum total protein concentrations in the control group were 2.44 ± 0.41 mg dL^{-1} and 3.88 ± 0.41 mg dL^{-1}, respectively. The total protein concentration significantly differed between the groups only 1 h after the challenge (Table). After the challenge, the plasma chloride concentrations increased in both groups at 3 h, but the mean chloride concentrations did not differ significantly at any sampling time. The plasma calcium concentrations in both the control and experimental groups relatively increased compared to the baseline (Table). However, a comparison between the experimental and control groups showed that there were no significant differences at any time point. The potassium ion concentration increased from 2.46 ± 0.34 to 4.45 ± 1.38 mm L^{-1} 1 h after the challenge in the experimental group, and this increase was found to be statistically significant ($P < 0.05$). Similarly, the potassium ion concentration also increased to 3.08 ± 0.66 mm L^{-1} in the control group. Six hours later, the potassium ion concentration was restored in both groups (Table). The sodium concentration increased from 133.82 ± 7.10 to 152.62 ± 0.95 mm L^{-1} in the experimental group due to the thermal challenge. Similarly, the sodium concentration also increased in the control group. In the control group, the lowest mean plasma phosphate level was 11.70 ± 0.81 mg dL^{-1} at 24 h, and the highest level was 15.98 ± 0.87 mg dL^{-1} at 48 h. In the experimental group, the lowest mean plasma phosphate level was 11.00 ± 0.61 mg dL^{-1} at 6 h while the highest

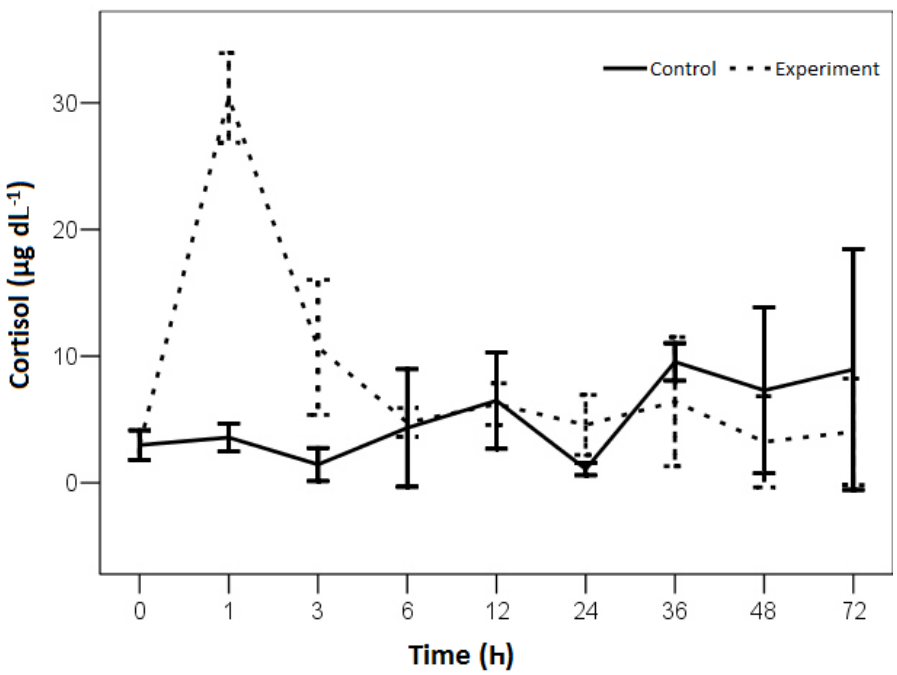

Figure 1. Cortisol level in the control and experimental groups after the acute thermal challenge. Error bars represent SE.

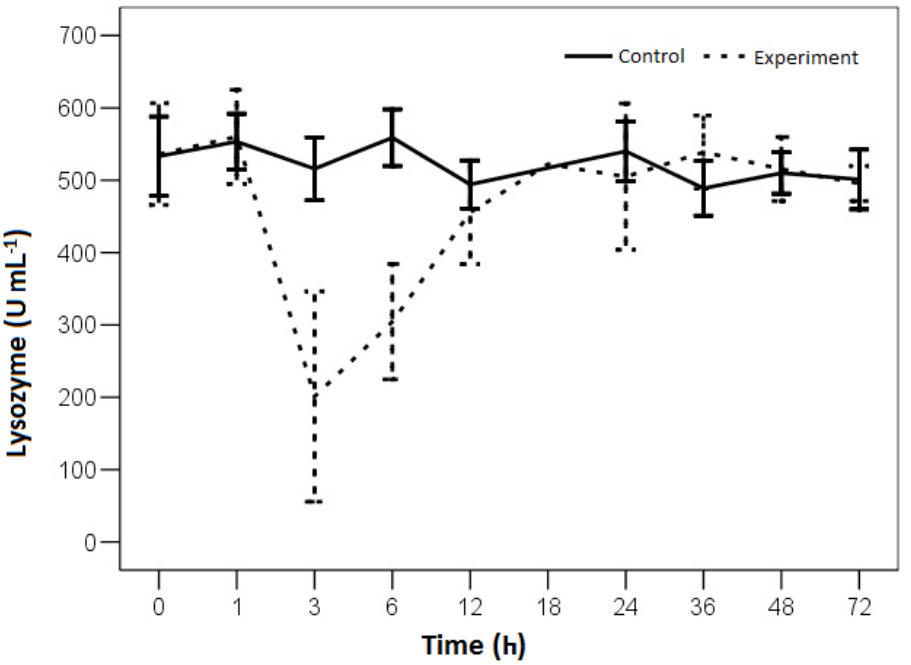

Figure 2. Lysozyme activity in the control and experimental groups after the acute thermal challenge. Error bars represent SE.

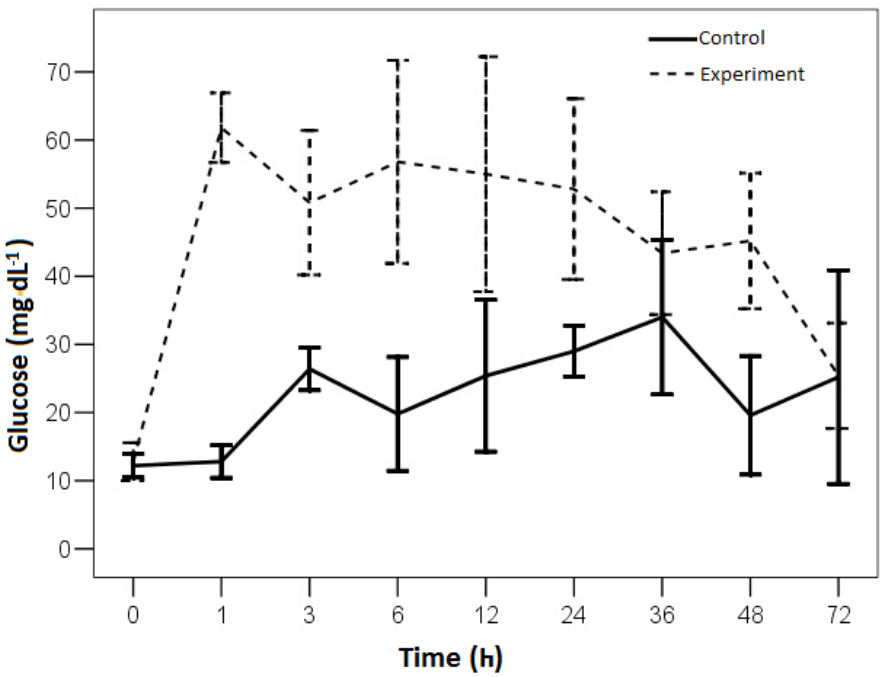

Figure 3. Glucose levels in the control and experimental groups after the acute thermal challenge. Error bars represent SE.

mean plasma phosphate level was 15.98 ± 0.81 mg dL^{-1} at 48 h. The differences in mean plasma phosphate levels were not statistically significant between the experimental and control groups during the experiment.

4. Discussion

Many studies have shown that the handling, confinement, vaccination, and grading of fish, as well as acute or chronic fluctuations of water temperature, cause a quick

Table. Serum ions and total protein concentration in Black Sea trout after the acute thermal challenge.

Variables	Groups	Time intervals (h)								
		0	1	3	6	12	24	36	48	72
TP (mg dL^{-1})	Exp.	2.44 ± 0.41^{ab}	3.20 ± 0.29^{bcde}	3.42 ± 0.13^{cde}	2.72 ± 0.16^{abc}	3.16 ± 0.16^{bcde}	3.64 ± 0.25^{de}	3.24 ± 0.25^{bcde}	2.88 ± 0.10^{abcd}	3.12 ± 0.36^{abcde}
	Cont.	2.44 ± 0.41^{ab}	2.30 ± 0.29^{a}	3.08 ± 0.10^{bcde}	3.50 ± 0.37^{cde}	3.30 ± 0.37^{bcde}	3.88 ± 0.20^{e}	3.10 ± 0.10^{bcde}	3.38 ± 0.20^{cde}	2.96 ± 0.11^{abcd}
Cl (mmol L^{-1})	Exp.	115.11 ± 1.71^{a}	126.56 ± 0.99^{bcde}	122.02 ± 3.57^{abcde}	123.32 ± 5.12^{abcde}	126.26 ± 4.02^{bcde}	131.86 ± 0.95^{bc}	132.98 ± 1.86^{e}	131.56 ± 1.26^{de}	118.90 ± 3.47^{abcd}
	Cont.	115.1 ± 1.71^{a}	118.3 ± 3.43^{abc}	129.2 ± 1.29^{cde}	120.36 ± 6.03^{abcde}	126.04 ± 0.89^{abcde}	128.04 ± 0.68^{bcde}	128.64 ± 0.88^{bcde}	132.26 ± 0.99^{e}	124.14 ± 5.13^{abcde}
Ca (mg dL^{-1})	Exp.	8.20 ± 0.37^{a}	11.30 ± 2.17^{bcd}	14.24 ± 1.17^{bcd}	9.58 ± 1.35^{ab}	13.30 ± 0.95^{bcd}	15.74 ± 1.67^{d}	13.62 ± 1.32^{bcd}	10.36 ± 0.79^{abc}	11.70 ± 0.65^{abcd}
	Cont.	8.20 ± 0.37^{a}	7.64 ± 0.53^{a}	12.02 ± 1.30^{abcd}	12.72 ± 1.65^{bcd}	11.46 ± 2.26^{abcd}	13.66 ± 1.75^{bcd}	11.86 ± 1.19^{abcd}	14.50 ± 1.33^{cd}	10.68 ± 1.04^{abc}
K (mmol L^{-1})	Exp.	2.46 ± 0.34^{a}	4.45 ± 1.38^{bc}	3.94 ± 0.93^{b}	3.73 ± 1.14^{ab}	2.25 ± 0.57^{ab}	1.73 ± 0.73^{a}	1.73 ± 0.73^{a}	1.69 ± 0.26^{a}	1.73 ± 0.28^{a}
	Cont.	2.46 ± 0.34^{a}	3.08 ± 0.66^{ab}	3.92 ± 1.08^{b}	3.93 ± 1.36^{ab}	2.45 ± 0.47^{ab}	1.47 ± 0.59^{a}	1.17 ± 0.59^{a}	2.36 ± 0.35^{a}	2.20 ± 0.15^{a}
Na (mmol L^{-1})	Exp.	133.82 ± 7.10^{a}	152.62 ± 0.95^{cd}	142.78 ± 6.04^{abcd}	146.94 ± 4.23^{abc}	155.66 ± 2.75^{abcd}	157.00 ± 1.88^{cd}	157.68 ± 1.88^{d}	155.68 ± 1.87^{cd}	156.28 ± 2.44^{cd}
	Cont.	133.82 ± 7.10^{a}	135.72 ± 3.77^{ab}	137.44 ± 6.34^{b}	144.60 ± 2.17^{ab}	147.88 ± 0.66^{abcd}	151.32 ± 1.16^{bcd}	151.32 ± 1.16^{cd}	156.14 ± 1.25^{cd}	147.00 ± 6.67^{abcd}
P (mg dL^{-1})	Exp.	12.20 ± 0.86^{ab}	13.16 ± 0.86^{ab}	11.80 ± 0.37^{ab}	11.00 ± 0.61^{a}	11.64 ± 0.24^{a}	12.24 ± 0.56^{ab}	12.24 ± 0.56^{ab}	15.98 ± 0.81^{b}	12.20 ± 0.33^{ab}
	Cont.	12.20 ± 0.86^{ab}	12.96 ± 1.11^{ab}	11.18 ± 0.63^{b}	12.34 ± 0.82^{ab}	12.86 ± 0.48^{ab}	11.70 ± 0.81^{ab}	11.7 ± 0.82^{ab}	15.98 ± 0.87^{b}	15.82 ± 1.14^{b}

TP: Total protein, Cl: chloride, Ca: calcium, K: potassium, Na: sodium, P: phosphate. Means in the same rows with different letters (a, b, c, d, e) differ significantly ($P < 0.05$). Data are expressed as mean ± SE.

and temporary increase in plasma cortisol levels, which leads to the development of primary stress response in fish (7,12,16,17). Secondary physiological responses lead to an increase in glucose levels for energy use and lysozyme, which is involved in the innate defense mechanism (8). Cortisol is the most important glucocorticoid secreted by interrenal kidney tissue in bony fish (18). Many researchers have reported that plasma cortisol levels increased in fish exposed to stress (8,19). In most fish, cortisol levels increase rapidly after stress and later return to initial levels within 6–48 h (20).

The impact of thermal shock and temperature fluctuations can vary depending on the fish species. Daily ±5 and ±1 °C temperature fluctuations caused no changes in the plasma cortisol levels of cutthroat trout (*Salmo clarki clarki*) (21) or Arctic charr (*S. alpinus*) (12). However, a daily 13.5 °C (from 6.5 to 20 °C) temperature change caused plasma cortisol levels to increase in Coho salmon (*Oncorhynchus kisutch*) (22). In our study, the serum cortisol levels rose nearly 10-fold within 1 h and decreased to the initial levels within 6 h in Black Sea trout that had been subjected to a 10 °C temperature increase (from 15 °C to 25 °C) for 30 min. Perez-Casanova et al. (23) reported that plasma cortisol levels increased in both 10-g and 50-g Atlantic cod (*Gadus morhua*) when the temperature rose from 16 °C to 24 °C (a progressive 2 °C h^{-1} increase). The cortisol level increased significantly within 1 h after 30 s of netting and decreased after 6 h in the Atlantic cod acclimated to 3 different temperatures (4, 10, and 14 °C) for 12 days (17).

Lysozyme hydrolyzes the peptidoglycan layer of the bacterial cell wall. Therefore, it is a very important component of the nonspecific humoral immune system (24). However, lysozyme activity in the blood serum of stressed fish shows constant change. Stressors such as handling, transport, or high ammonia levels inhibited the lysozyme activity in rainbow trout (*Oncorhynchus mykiss*), the activity returning to normal values after 24 h (25). Reduced lysozyme activity has been reported in dab (*Limanda limanda* L.) due to transportation stress (26). In our study, the lysozyme activity decreased to 201.11 ± 72.67 U mL^{-1} at 3 h after acute thermal stress and returned to a normal level (532.66 ± 36.50 U mL^{-1}) 24 h later. However, an increase in the serum lysozyme activity was reported in rainbow trout (*O. mykiss*) exposed to acute stress such as transport, handling, and compression (27).

The plasma glucose level was greater in the experimental group than in the control group 1 h after acute thermal stress. The highest glucose concentration (up to 61.8 ± 2.55 mg dL^{-1}) was reached within 1 h, and it started to decline at 24 h. The glucose concentration decreased to the control group level (25.4 ± 3.86 mg dL^{-1}) at 72 h. Biswas et al. (28) reported similar results in red sea bream (*Pagrus major*). Acute stress caused an increase in the glucose level in red sea bream; the level began to decline 24 h later and returned to the normal level 72 h later. Kubilay and Uluköy (27) reported increasing amounts of serum glucose in rainbow trout (*O. mykiss*) after acute stress (transport, screening, and compression).

Plasma total protein levels, as well as chloride, sodium, calcium, and potassium ion concentrations, somewhat increased in the experimental group after acute thermal stress. However, only phosphate ion concentrations differed between the groups and time points. The total

protein level decreased to the normal level 6 h later. The calcium and potassium concentrations also decreased to normal levels after 6 h. Sodium concentrations increased in both the experimental and control group. This increase may have been caused by the handling stress. Although the plasma sodium concentration fluctuated around the initial concentration during 72 h, the chloride ion concentration returned back to its normal level at 72 h (Table). Lyytikäinen et al. (12) studied plasma ion concentrations of Arctic charr (*S. alpinus*) acclimated to different water temperatures (10.3, 14.1, and 18.1 °C) and to fluctuating temperatures. After acute stress, sodium and chloride concentrations increased, but the potassium concentration decreased in Arctic charr at a temperature of 18.1 °C. Prosser et al. (29) also reported higher sodium, calcium, and potassium ion concentrations in goldfish acclimated to higher temperatures. In fresh water, an increased cortisol level can stimulate chloride cells and Na^+/K^+-ATPase, and this interaction may cause the ion concentrations to increase with the increase in water temperature (30).

In conclusion, stressed Black Sea trout (*S. trutta labrax*) recovered from acute thermal stress within 3 days. The levels of cortisol, glucose, and lysozyme activity were good indicators for the determination of acute stress. Further research should be conducted on the effects of chronic thermal stress and lower–upper thermal limits of Black Sea trout.

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