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First Use of Benzocaine as an Anesthetic in Black Sea Salmon (*Salmo labrax*) Fry

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Abstract

This study aims to find effective concentration and lethal dose of benzocaine in Black sea salmon and determine effect of exposure time to air on survival rate of fish. In the study total 600 fish used with 3.02±0.14 cm and 0.29±0.03 grams. Fresh water for trout rearing used and water temperature was 11.4±0.66°C during experiment. In order to determine the effective anesthetic dose of benzocaine in Black Sea salmon 20 fry were treated with benzocaine solutions of 20, 25, 30, 35, and 40 mg/l. To determination of lethal concentration five benzocaine concentration tested as 60, 70, 75, 80, and 85 mg/l. fry were simultaneously released into 28 mg /l consecration benzocaine solution to determine effect of exposing fish to air. After being kept in an anesthetic substance for 3 minutes, all fish were taken on fish net at the same time and the fish were exposed to air for 5, 10, 15, 20 minutes respectively, and they were taken to separate tanks according to their exposure time to the air and observed for 24 hours, mortality were noted. Effective concentration and lethal dose were determined by Probit analysis. As a result, this study showed that 25-26 mg/l benzocaine is sufficient to anesthesia Black sea salmon and approximately 3 times of this dose is lethal for these fish. Also, it showed that air exposure after anesthetic use is very critical in salmon fish fry. After from 10 minutes 15 to 20 minutes of air exposure considerable fatalities occurred. Survival rates were 80 to 90 % between 5 and 10 minutes of air exposure however, when fish leave in air for 15 minutes and 20 minutes this rate fall drastically to below 20 % survival rate within first 24 hours.

Introduction

Handling fish without causing too much damage is quite important, stress from manipulative procedures can cause a large number of mortalities, especially carrying a high density of fish in long-distance transit (Smit and Hattingh, 1979). Several chemicals have been used for the anesthetization of fish (Bolasina et al. 2017). Tricaine methane-sulfonate (MS-222) is one of the widely used anesthetics for handling fish, yet the withdrawal period of MS-222 can create restrictions (Gilderhus,1989). MS-222 is a water-soluble substance usually used buffered with sodium bicarbonate to reduce acidity, and applying method is usually water baths (Bolasina et al. 2017). Clove oil is a relatively new anesthetic in fish handling, mainly extracted from *Eugenia aromaticum* (i.e. Syzygium aromaticum) or *Eugenia caryophyllata* plants (Ross and Ross, 2008), and active ingredients are eugenol (76.8-88.58%), eugenyl acetate (1.2- 5.62%) and β-caryophyllene (1.39-17.4%) (Jirovetz et al., 2006; Chaieb et al., 2007). Akbulut et al. (2012) found that clove oil in sturgeon fish fry (*Acipenser baerii* Brandt, 1869) is as effective as MS-222. Benzocaine is a chemically very similar substance to MS-222. It is a white, odorless, tasteless crystalline ester of p-aminobenzoic acid and ethanol, and as it does not have the MS-222 sulphonyl side-group, it is almost insoluble in water (only 0.04% w/v) and must first be dissolved in either acetone or ethanol. The standard approach is to prepare a stock solution of the solvent, usually 100 g/l, which, if kept in a dark, stoppered bottle, will stay for long periods (Rose and Rose, 2008). Dawson and Gilderhus (1979) carried out a testing experiment on five fish species and found that benzocaine is creating a similar effect to MS-222; these series of tests showed that benzocaine is effective and safe to use on salmonid species both small and large fish.

Expected characteristics of anesthetics are fast effect and recovery times, without toxic effect (both fish and humans), fast elimination from the body, non-permanent physiological effect, high solubility in fresh and saltwater, and cost-effective (Schoettger and Julin, 1967; Treves-Brown, 2000; King et al., 2005).

Salmo labrax (Pallas, 1814) commonly known as black sea salmon is anadromous fish that widely spread species in the Black sea basin (Kottelat and Freyhof, 2007). The culture of black sea salmon is rapidly developing in Turkey (Sahin, et. al., 2007). The culture of the black sea salmon is supported by the Republic of Turkey, Ministry of Agriculture and Forestry (Çoban et. al., 2020) and in 2020, 1800 tons of Black sea salmon were produced in Turkey (TÜİK., 2020). Despite anesthetics used in other salmonid species being common, anesthetic trials in black sea salmon are very limited, especially the effect of different dosages of the anesthetic on different sizes and ages Black sea salmon is scarce. Thus, this study aims to find an effective benzocaine dosage in black sea salmon fry.

Material and Method

The experimental fish: The study was carried out at recirculating aquaculture systems in Trabzon Central Fisheries Research Institute. The 600 fry of black sea salmon fry, which appeared healthy before the experiment, were placed in 300-liter tanks with continuous water flow for 24 hours before. The fry was not fed during this period.

Experimental Procedure

Three separate experiments were performed to determine the effective concentration, effect of air exposure on anesthetized fish, and the lethal concentration of benzocaine.

Experiment 1: Determining the effective concentration

To determine the effective anesthetic dose, the black sea salmon fry were treated with benzocaine solutions of 20, 25, 30, 35, and 40 mg /l, and the loss of balance in the fish were observed and noted for the duration of anesthesia. Subsequently, the length and weight of the fish were measured, and the fish were immediately released into anesthetic-free water, and their first mobilization and recovery times were noted. The fish were taken into different tanks according to the anesthetic doses applied and the mortality of the fish was observed for 24 hours.

Experiment 2: Effect of air exposure

Twenty salmon fry were simultaneously released into 28 mg/l of consecration benzocaine solution. After being kept in an anesthetic solution for 3 minutes, all fish were taken on a fish net at the same time and the fish were exposed to air for 5, 10, 15, and 20 minutes respectively. Then, they were placed in separate tanks according to their exposure time to the air, and mortality was observed for 24 hours, mortality was noted.

Experiment 3: Determination of lethal concentration

Ten fish were taken each time and released in 60, 70, 75, 80, and 85 mg / l benzocaine solutions. The fish were kept in an anesthetic solution for 3 minutes and their loss of balance was noted. Subsequently, the fish exposed to different anesthetic doses were placed in separate tanks with water without anesthetic. The first mobilization, normal swimming times, and mortality rates were observed within 24 hours.

Water Quality Parameters

Before, during, and after the experiment, temperature, pH, dissolved oxygen, and conductivity were measured using a digital multipurpose measuring device (HACH HQ40d) using the methods described by APHA (1985).

Statistical Analysis

The data were analyzed using SPSS v16 and Excel package programs. Normality analysis of the results of waking up, fainting, and death rates were performed using the Shapiro-Wilk test. Kruskal-Wallis test at a 0.05 significance level was used to determine the level of difference between groups. Mann-Whitney comparison test was used to determine the difference between the test groups at a 0.05 significance level. Percentage data such as survival and mortality rates were transformed to arcsine before statistical analysis. The effective dose and lethal dose were determined by Probit analysis.

Results

In this study, three different experiments were performed to determine the response of black sea salmon fry to anesthesia. The average length and weight of the fish used in the study were measured as 3.02±0.14 cm and 0.29±0.03 grams, respectively. The results made to determine the effective benzocaine concentration are given in Table 1. The Kruskal-Wallis test performed to determine the difference between induction and recovery times at different concentration levels, showed that the difference between the induction times (I2; X^2 = 34.45, P = 0.00 <0.05) was found significant, while there was no difference between the recovery times (R2; X^2 = 0.72, P = 0.125> 0.05). The multiple Mann-Whitney comparison test showed that the average syncopal time at 20 mg /l concentration was longer than the other concentrations (P = 0.00 <0.05).

The results collected in the experiment of exposing the fish to the air outside of the water after completely syncopal are given in Figure 1. When the fry of black sea salmon fry is kept out of the water for more than 10 minutes, it was observed that there was over 90% mortality in the first 24 hours. In the statistical analysis, it was observed that there was no difference between 5 minutes and 10 minutes (P=0.38), and between 15 minutes and 20 minutes (P=0.38).

In the experiment conducted to determine the lethal dose of benzocaine for black sea trout fry, it was determined that deaths started at a concentration of 70 mg / l and 95% of the fish exposed to concentrations of 80 mg /l and above died within 24 hours. In the analysis, it was observed that there was no difference between 80 mg and 85 mg (P=0.12). The Lethal Dose of benzocaine concentration for Black Sea salmon fry was calculated as LD_{50} = 75.7 mg / l at a 95% confidence interval (Figure 2).

Discussion

In recent years, Black sea trout farming has started to grow rapidly in line with the increasing demand. The handling of this fish requires to be transported alive and subjected to other processes, so the use of the most effective anesthetic in the right dose becomes very important (Ross and Ross, 2008). Living things can give different reactions to anesthetic agents, therefore, in anesthetic administration studies, individuals should be closely monitored and an average time and dose should be determined by their reactions (King et al., 2005). This study provides information about the appropriate dose time and method of administration. The study has been carried out under Recirculating aquaculture systems (RAS) conditions. The water used in this study was ideal for trout and was kept under BSGM (2004) Trout fish Aquaculture water criteria. For this reason, it is assumed that the water used in experiments is no effect on the results of this study. As seen in Table 2, water quality criteria are suitable for salmon farming.

In aquaculture, weighing, scaling, tagging, microinjections, and carrying between tanks can cause injuries and stress and result in death. Anesthetic agents are used to preventing these injuries and stress. Also, the effective concentrations of anesthetic substances

Table 1. The results of induction and recovery times at different benzocaine concentrations for the estimation of effective dose (I: Induction time and R Recovery time).

	Length - Weight		Induction time (minutes)		Recovery time (minutes)	
Dose (mg/l)	Length (cm)	Weight (gr)	I1	I2*	R1	R2
20	2.79	0.26	5.21	7.35[a]	1.35	2.25
25	3.08	0.28	3.12	4.22[b]	1.21	2.43
30	3.04	0.29	2.13	3.27[b]	2.06	2.46
35	3.04	0.27	2.25	3.39[b]	1.34	2.38
40	3.15	0.33	1.46	2.19[b]	1.05	2.43

*Different superscripts within a column indicate statistically significant differences between means (p≤0.05).
The effective benzocaine concentration for black sea salmon fry was calculated as EC_{50} = 25.82 (22.93 - 28.52) mg / l at 95% confidence interval.

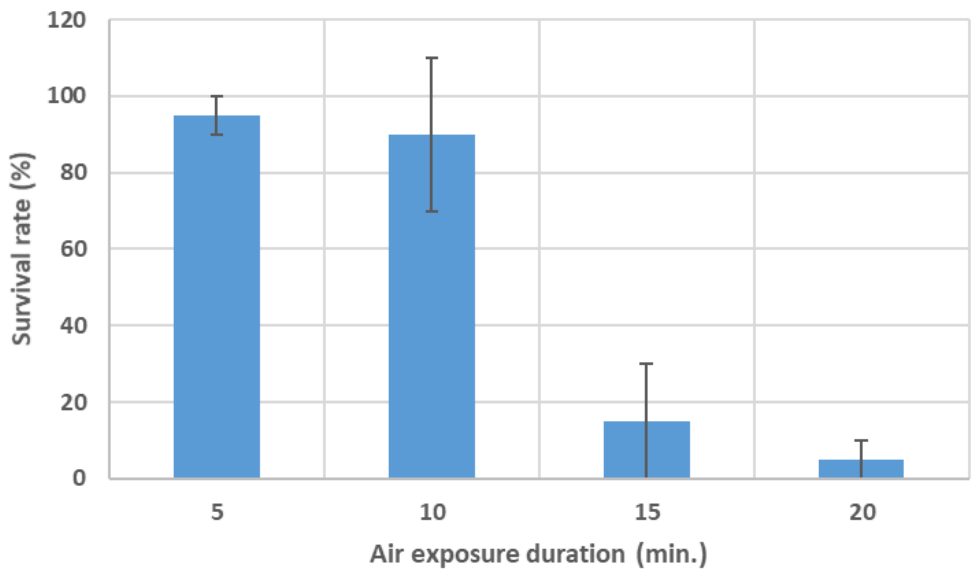

Figure 1. Survival rates of black sea salmon fry when taken out of the water at different air exposure times.

may differ according to the fish species and the characteristics of the anesthetic substance (Priborsky and Velisek, 2018). The studies regarding anesthetic application in Black sea salmon (*S. labrax*) are limited. However, there are several studies on other salmonid species.

For example, the effective dose of MS-222 for rainbow trout has been reported as 100mg / l (Soivio et al., 1977). Similarly, for *Oncorhynchus mykiss* 30-50 mg/l (Oswald, 1978), for *Salmo salar* (smolts) 40 mg (Ross and Ross, 1984), and 40 mg/l were reported as benzocaine doses for *S. trutta* (Oswald,1978). This dosage of 25.8 mg/l for Black sea salmon fry is lower compared with recommended values for other salmonid species.

On the other hand, fish are anesthetized to reduce injury and stress caused by aquaculture activities. At the same time, since some of the above-mentioned aquaculture activities cannot be carried out in the water, the fish must stay out of the water for a certain period of time and have contact with the air during these activities. Cassiano et al. (2012) reported that when they keep *Trachinotus carolinus* larvae, a marine fish, out of the water, the survival rate changes depending on the feeding of the larva, and the survival rate is 54% when *T. carolinus* larvae are kept out of the water for 3 minutes under standard feeding conditions. In this study, it was found that 90% of the juvenile fish were alive if the fish were exposed to air for up to 10 minutes and this period is considered a suitable time for measuring the length and weight of the fish, marking, photographing or any other process. Just as these studies provided, our study showed that when the fry of Black sea salmon was kept out of the water for more than 10 minutes, there was over 90% mortality in the first 24 hours.

Gilderhus and Marking (1987) compared 16 different anesthetics including benzocaine in rainbow trout and they found that four (benzocaine, MS-222, quinaldine sulfate, and 2-phenoxyethanol) out of the 16 chemicals met the criteria for efficacy. They also found that benzocaine showed rapid induction and rapid recovery time and was effective even at very low concentrations (35 mg/l).

To find the lethal and effective dose of benzocaine 10 different dosages had been used (effective dose 20, 25, 30, 35, 40, and lethal dose 60, 70, 75, 80, and 85 mg /l) and the fish were exposed to air for 5, 10, 15, 20 minutes respectively and they were taken to separate tanks according to their exposure time to the air and mortality was observed for 24 hours. The effective dose of benzocaine for Black sea salmon fry was found to be EC_{50} = 25.82 (22.93 - 28.52) mg / l and a lethal dose with a 90% mortality rate within 24 hours was found to be LD_{50} = 75.7 mg / l. On the other hand, Ferreira and friends (2020) carried out a study that uses benzocaine and methanol as anesthetics in African cichlid (*Aulonocara nyassae*) juveniles. They used two groups of fish and concentrations between 75 and 125 mg/L for

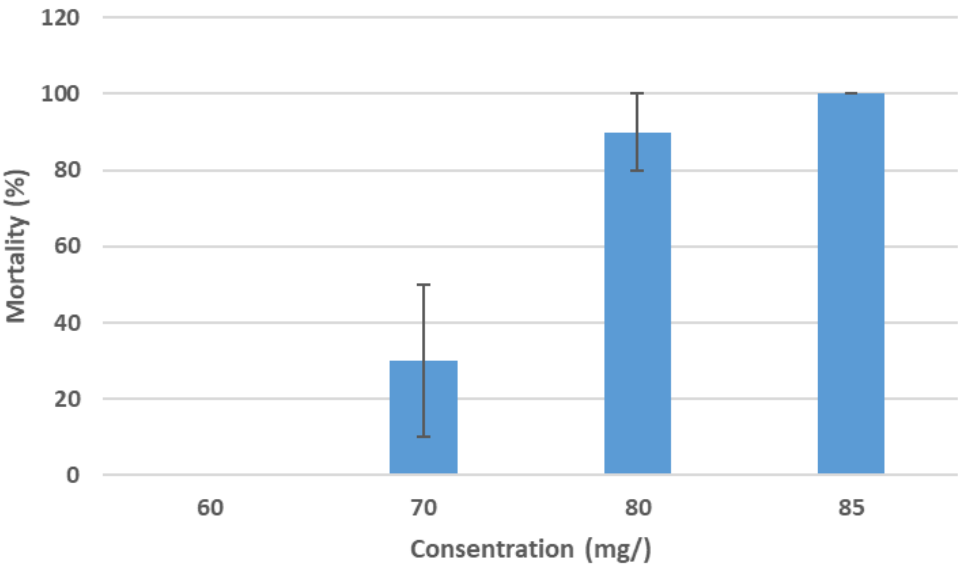

Figure 2. The mortality rate of black sea salmon fry at different benzocaine concentrations.

Table 2. Quality parameters of the water used in the experiment.

Parameter	Average ± SD
Temperature (° C)	11.40±0.66
pH	6.92±0.11
Dissolved Oxygen (mg / l)	10.10±0.54
Conductivity (µS / cm)	245±2.45

*SD: Standard deviation

group one and between 50 and 125 mg/l for group two and they found out that the first group showed high survival (> 90%) rate after 24 hours.

Conclusion

In conclusion, this study showed that 25-26 mg/l benzocaine is sufficient to anesthetize black sea salmon fry, and approximately 3 times this dose is lethal for black sea salmon fry. Also, it showed that air exposure after anesthetic use is very critical in salmon fish fry. After 10 minutes (i.e. 15 to 20 minutes) of air exposure, considerable fatalities occurred. Survival rates were 80 to 90 % between 5 and 10 minutes of air exposure however, when fish were exposed to air for 15 minutes and 20 minutes, the survival rate fell drastically to below 20 % within the first 24 hours.

Ethical Statement

This study was carried out with the approval of the animal experiments local ethical committee of the Fisheries Central Research Institute, dated 13.10.2020 and numbered 325.04.02/7.

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Author Contribution

ANS conceptualization, methodology, data collection, writing - review and editing, BA methodology, writing and review, data analysis, EA data collection, and writing, EK review, and editing

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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