# STUDIES OF THE PECULIARITIES OF SEA TROUT RESPONSES TO ENVIRONMENTAL STRESS FACTORS

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## ISPITIVANJE OSOBENOSTI REAGOVANJA MORSKE PASTRMKE NA STRESNE FAKTORE SREDINE

***Abstrakt***

Cilj rada je bio da se ispitaju uticaji dva izdvojena faktora sredine i njihovog kombinovanog delovanja na morsku pastrmku, procenom promena u morfofiziološkim, fiziološkim, imunološkim i mikrobiološkim parametrima kod riba kao odgovor na stres. Primenjene su višestruke istraživačke metode. Nađene su promene u pomenutim parametrima kod mlađi morske pastrmke na stupnju aktivne ishrane pod uticajem pojedinačnih stres faktora (gustina riba, protok vode) i pod njihovim kombinovanim delovanjem. Kombinovano delovanje dva stres faktora sredine u poređenju sa pojedinačnim uticajima izazvalo je mnogo značajnije promene u većini ispitivanih parametara.

***Ključne reči**: morska pastrmka, stresni faktori sredine,kombinovano delovanje*

## INTRODUCTION

In intensive fish culture, different environmental stress factors affect the fish organism. Deleterious stimuli such as temperature changes, decrease in oxygen concentration and increase in ammonia concentration in water induce stress reaction in fish (P i c k e r i n g, 1993; K r o u p o v a et al. 2005). In turn, depending on the magnitude of stimuli, a stress reaction, which is an integrated response with behavioural, neural, hormonal and physiological elements of the fish organism, can worsen the fish health

status and reduce the organism‘s resistance to diseases (I w a m a et al. 1995; K a z l a u s k i e n ė et al. 2004). Some of diseases, such as fin necrosis, are frequently observed in hatchery practice, however, specific causes of this infection and pathogens have not been ascertained yet. The aim of the study was to investigate and compare the affects of different single stress factors and their combined action on sea trout (*Salmo trutta trutta* L.) evaluating morphophysiological, physiological, immune and microbiological parameters.

## MATERIALS AND METHODS

Sea trout fry (Q – 2.82 ± 0.12 g; L – 6.45 ± 0.69 cm) were brought from the Žeimena Salmon Hatchery (Švenčionys district, Lithuania). Fish were reared in the semi-recirculated system constructed according to the guidelines by M o r t e n s e n et al. (2000). The system consisted of four 45 l capacity aquariums, two buffer tanks, a submersible water pump and PVC piping. The total water volume in the system was 0.81 $m^3$. One of the buffer tanks was loaded with 0.25$m^3$ Bio-Blok® 200 (Expo-Net, Denmark) filter media to serve as a biological filtration unit. A high water exchange level was kept constantly at 3000 l of fresh water per one kilogram of feed added to the system. This maintained adequate water temperature and allowed a higher feed input. Four groups of visually healthy fish were investigated: group I (control) – (150 specimens) fish density in the aquarium – 10 kg/$m^3$ and water flow – 1.4 l/min; group II – (150 specimens) fish density – 10 kg/$m^3$ and water flow – 0.79 l/min; group III – (300 specimens) fish density – 20 kg/$m^3$ and water flow – 1.4 l/min; group IV – (300 specimens) fish density – 20 kg/$m^3$ and water flow – 0.79 l/min. A total of 900 individuals were studied. Sea trout were fed with Aller Aqua Futura (Denmark) extruded fish feed. The amount of the feed given was calculated as a percentage according to fish biomass in the aquarium based on recommendations by the producer. The duration of the experiment was 20 days. The specimens were analysed (20 individuals from each aquarium) at the end of the test. The experiment was performed using artesian water. The average hardness of water was approximately 284 mg/l as $CaCO_3$, alkalinity was 244 mg/l as $HCO_3^-$, mean pH was 7.2–7.8, temperature was 15.0 ± 0.5°C, and oxygen concentration ranged from 8 to 10 mg/l. The morphophysiological state of sea trout fry was evaluated using morphophysiological parameters: the condition factor (CF) and the liver somatic index (LSI). Physiological (haematological) analysis was performed, and erythrocyte (Er, $10^6 \times mm^{-3}$) and leukocyte (Leu, $10^3 \times mm^{-3}$) counts were evaluated using routine methods (S v o b o d o v a, V y k u s o v a, 1991). Glucose concentration (Mmol/l) was determined with „EKSAN-G“ (K u l y s et al. 1989). Caudal fin specimens were taken from groups I, III and IV of sea trout fry for the detection of fin necrosis causing bacteria. Caudal fins were weighed, homogenized and diluted with a buffer solvent (pH7.3). Bacterial cells were separated from fins by serial centrifugation according to Holben (H o l b e n, 1997). For identifications of bacteria, we used the following molecular techniques: genomic DNA isolation was performed using a kit (Genomic DNA Purification Kit, MBI Fermentas); amplification of the bacterial 16S rRNA gene (700 bp) was performed using the universal bacterial primers w010 340F (5’-ACT CCT ACG GGA GGC AGC A-3’) and w007 1100R (5’-CTC GTT GCG GGA CTT AAC-3’) (S k r o d e n y t ė-A r b a č i a u s k i e n ė et al. 2006); PCR products were extracted using a Cyclo-pure gel extraction kit (Amresco, USA); PCR products cloning was performed using a Gene-

JET™ PCR Cloning Kit (MBI Fermentas); transformation of competent *Escherichia coli* DH5α cells was performed using a TransformAid™ Bacterial Transformation Kit (MBI Fermentas); recombinant plasmids containing appropriate insert isolations were performed as described by B i r n b o i m et al. (1979); DNA sequence analysis was performed on an ABI Prism model 377 automated DNA sequencer ABI (Foster City, CA). The obtained sequences were compared to sequences from the Gene Bank. Phylogenetic sequence analysis was performed using the CLC Free Workbench version 0.91 software (http://www.clcbio.com). Differences between the measured characteristics were tested by Student's t-test ($p < 0.05$) using the programme GraphPAD InStat (USA).

## RESULTS AND DISCUSSION

***Morphophysiological parameters.*** No significant differences in the LSI (1.15 ± 0.12) and CF (1.03 ± 0.03) of sea trout fry were recorded in group II (water flow twice reduced) as compared with the control group –(1.17 ± 0.13; 1.05 ± 0.03, respectively). The LSI of fry estimated in group III (fish density twice enlarged) was significantly ($p < 0.0001$) higher (1.56 ± 0.14) than that in the control group (1.17 ± 0.13). However, no significant differences in the CF of sea trout fry were recorded in group III (1.03 ± 0.04 1.05 ± 0.03) as compared with group I (1.05 ± 0.03). The LSI of fry recorded in group IV (water flow twice reduced and fish density twice enlarged – combined action) was significantly ($p < 0.0001$) higher (1.61 ± 0.12) and the CF was significantly ($p < 0.0001$) lower than those in the control group (1.17 ± 0.13; 1.05 ± 0.03, respectively). In intensive fish culture, the effects of different factors such as temperature, oxygen and ammonia concentration in water, as well as stocking density and food accessibility, are responsible for a decrease in the growth of fish (L i e, 2001). Moreover, according to scientific data different morphophysiological parameters are rather sensitive and adequately reflect the negative impact of changing environmental factors on the developing and growing organism (K a z l a u s k i e n ė et al. 2004; F r o e s e, 2006).

***Physiological, immune parameters.*** The erythrocyte and leukocyte counts significantly ($p < 0.05$) increased ($1.71 \pm 0.14\ 10^6 \times mm^{-3}$ and $16.45 \pm 1.3\ 10^3 \times mm^{-3}$, respectively) in the blood of group II fish as compared to control fish ( $1.29 \pm 0.17\ 10^6 \times mm^{-3}$ and $12.86 \pm 1.410^3 \times mm^{-3}$, respectively). An increase in the number of fish in group III induced a significant elevation in erythrocyte ($1.88 \pm 0.12\ 10^6 \times mm^{-3}$) and leukocyte counts ($29.8 \pm 3.3\ 10^3 \times mm^{-3}$) as compared to the same parameters in the control group. All studied fish parameters induced by the combined action of two stress factors (group IV) were significantly different from those in the control group. The erythrocyte count ($1.65 \pm 0.70\ 10^6 \times mm^{-3}$) and leukocyte count ($25.9 \pm 1.5\ 10^3 \times mm^{-3}$) were significantly elevated, whereas glucose concentration (1.90 ± 0.1 Mmol/l) was significantly reduced (control group 2.7 ± 0.2 Mmol/l). These data are in accordance with the results obtained by I w a m a et al. (1995) about changes in the blood of salmon juveniles induced by human activities and the worsening of water quality. Meanwhile, when stress stimuli were prolonged, the amount of erythrocyte and haemoglobin concentration decreased, the processes of leukopoiesis were suppressed and fish immune resistance was reduced (V o s y l i e n ė et al. 1999). According to V o s y l i e n ė (1996), glucose concentration in the blood of fish exposed to long-term stress is reduced to the undetectable level.

***Microbiological parameters.*** After cloning, 55 bacterial clones were isolated randomly from plates: 20 from each of groups III and IV and 15 from the control group.

Uncultured γ-proteobacteria (5 of 15), *Aeromonas* (4 of 15) and unclassified Cyanobacteria (4 of 15) were isolated from the control group of sea trout fry fin samples. Meanwhile, *Aeromonas* (8 of 20) and uncultured γ-proteobacteria (4 of 20) were detected in group III fin samples, and *Aeromonas* (14 of 20) and *Pseudomonas* (5 of 20) in group IV fin samples. Phylogenetic analysis of *Aeromonas* strain sequences showed that 19 and 23 clone sequences from group III, as well as 16, 17, 18, 20 and 21 clone sequences from group IV, were closest to *Aeromonas hydrophila.* The other sequences were closest to *Aeromonas* sp. sequences (Fig. 1). Phylogenetic analysis of *Pseudomonas* strain sequences showed that the universal bacterial primers w010 340F and w007 1100R for the amplification of the bacterial 16S rRNA gene were insufficient to separate the species in the same genus. The tested bacterial sequences may belong to *Pseudomonas fluorescens* and *Pseudomonas* sp. Most of these bacteria were detected in group IV, with combined action of the two environmental stress factors. As opportunistic pathogens, *Aeromonas hydrophilia* and *Pseudomonas* are abundant in all aquatic environments, however, fish fall ill when environmental hazards lead to changes in their physiological state and the weakening of the immune system. Under the combined action of several environmental stress factors, opportunistic bacteria can become primary pathogens with tremendous reproductive potential leading to high numbers of infected fish and possibly to serious losses in fish hatcheries.

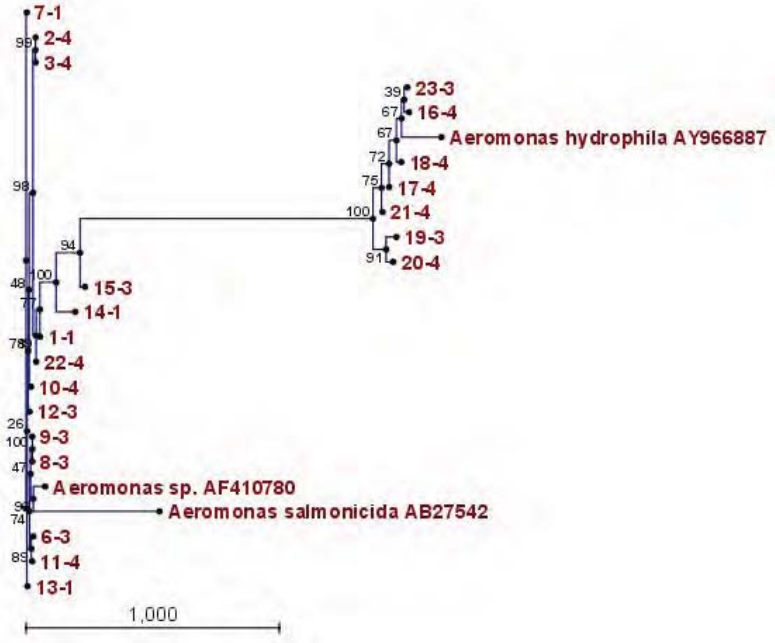


**Figure 1.** Phylogenetic tree showing the relationships among *Aeromonas* based on 16S rRNA gene sequences isolated from the tail fin of sea trout (numbers indicate clones) as compared to sequences of the same gene of the known bacterial genera and species derived from the Gene Bank. The scale bar of the bootstrap consensus tree represents genetic

distance (substitutions per 100 nucleotides). The tree was constructed using the neighbor-joining analysis of a distance matrix obtained from a multiple-sequence alignment. Bootstrap values (expressed as percentages of 100 replications) are shown at branch points.

## CONCLUSIONS

1. No significant differences in the LSI, CF and glucose concentration, and significantly elevated erythrocyte and leukocyte counts in the blood of sea trout fry were recorded in group II (water flow twice reduced) as compared to those found in the control group.
2. The LSI, and erythrocyte and leukocyte counts were significantly elevated and no significant differences in the CF and glucose concentration were recorded in group III (fish density twice enlarged). Fin necrosis causing bacteria was detected in group III: *Aeromonas hydrophilia* – 2 strains and *Pseudomonas* – 2 strains, while in group I they were absent.
3. Combined action of the two environmental stress factors (water flow twice reduced and fish density twice enlarged) caused more significant alterations in all studied parameters of sea trout fry (group IV). The largest number of opportunistic pathogens (*Aeromonas hydrophilia* – 5 strains, *Pseudomonas* – 5 strains) was detected in group IV.
4. Under the combined action of several environmental stress factors, opportunistic bacteria with tremendous reproductive potential can become primary pathogens and can cause disease outbreaks in the fish hatchery.

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