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# INFLUENCE OF DIET CHANGES ON THE CONDITION AND PHYSIOLOGICAL STATE OF JUVENILE SEA TROUT (*SALMO TRUTTA*)*

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**Abstract**

**The aim of the study was to determine the influence of diets (factor D) and the time period (factor T) during which they were applied on the growth performance and physiological condition (blood plasma hematological and biochemical indicators of stress and immunity) in juvenile sea trout (*Salmo trutta*; initial body weight approximately 73 g). The diet of the fish that was used prior to the experiment (formulated feed; initial fish sample) was modified as follows: a different formulated feed (group B), mixed feed (feed B + prey fish; group B/N), prey fish exclusively (group N). The fish from group A were given the feed that was used prior to the beginning of the experiment. During the 28-day trial neither factors D nor T influenced absolute or relative fish growth rates. Factor D significantly influenced hematological indicators and leukograms, while the phagocytic index and cidal ability were determined by the time test (factor T). Myeloperoxidase ($A_{MPO}$) was related significantly with the influence of factors D and T and also with the interaction of D×T. A significant decrease in $A_{MPO}$ was noted after two weeks of the test in groups B, N, and B/N, but after four weeks this indicator did not differ from that confirmed in the initial fish sample. The factors tested influenced stress indicators, i.e., cortisol (D and T) and glucose (D). Significant increases in cortisol (group B) and glucose (groups A, B, and B/N) concentrations were noted after two weeks of the test. After the subsequent two weeks (four weeks of the test) these indicators also stabilized. Sea trout from aquaculture readily accept prey fish; however, changes in diet cause stress reactions such as temporarily reduced immunity. Thus, the procedure for preparing sea trout stocking material for release, which is to change the feed from formulated to natural (live fish), should last approximately four weeks.**

**Key words: fish welfare, food replacements, biochemical indicators, immunity, prey fish**

The abundance of anadromous salmonid populations in the Baltic Sea catchment area is in drastic decline (HELCOM, 2011, 2021; Dębowski, 2018). The causes of this include artificial barriers, river fragmentation preventing spawning migrations, pollution resulting in siltation and the destruction of natural spawning grounds, and overfishing during spawning migrations (van Puijenbroek et al., 2018). Detailed reports on and analyses of this situation refer to two species – Atlantic salmon (*Salmo salar*) and sea trout (*Salmo trutta*) (HELCOM, 2011). One method of active sea trout protection is stocking with material produced in aquaculture facilities that maintain broodstocks and supplementing them with wild spawners caught during spawning migrations (Dębowski, 2018). Cultured fishes are generally characterized by lower vitality than wild species, and the efficiency of their natural reproduction is also lower (Costas et al., 2013; Christie et al., 2014). Often the effectiveness of stocking cultured sea trout is low, and high fish mortality following release into natural water courses is explained by, inter alia, changes in foraging behavior (from the formulated feeds used in aquaculture to natural feed) and the inability of cultured fish to forage under natural conditions (Czerniawski et al., 2011). Currently, methods are being researched for naturalizing cultured fish for stocking that will permit, for example, developing the abilities of such fishes to search for natural feed, and, as a result, improving their adaptability to natural conditions and increasing survival rates (Brown and Laland, 2002; Donadelli et al., 2015; Krepski and Czerniawski, 2019; Yang et al., 2020).

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Producing stocking material under controlled conditions can decrease the adaptability of these fishes to natural environmental conditions, which can determine the effectiveness of stocking (Brown and Day, 2002; Brown and Laland, 2002; Donadelli et al., 2015). With salmonids, it is assumed that only approximately 15% of the fish released will achieve sexual maturity (Beamish et al., 2010; Irvine et al., 2013); however, this indicator often does not exceed 5% (McNeil, 1991), and the highest stocking material mortality is observed immediately following release into natural waters (Olla et al., 1998). This might be a consequence of the lower immunity of fish from hatcheries, poorly developed anti-predatory behaviors, and changes in foraging behaviors (Manassa and McCormic, 2012). Formulated feeds that are often supplied *ad libitum,* at the same places (using feeders), and at strictly controlled times do not encourage the development of the natural foraging skills that are required for survival under natural conditions. Fast fish growth rates, which are desirable under hatchery conditions, do not contribute to the formation of traits that are required under natural conditions in which it is important to forage over large areas (Saikkonen et al., 2011). Providing fishes in hatcheries with different diets and including live feed in the period preceding stocking can change foraging behavior and result in higher fish survival following release into natural water courses (Brown and Day, 2002; Donadelli et al., 2015). Studies conducted on Atlantic salmon indicated that these fish learn appropriate behaviors through observation and interactions with other individuals (Brown and Laland, 2002; Brown et al., 2003). It has also been demonstrated that exposing fishes to enriched rearing conditions (live feed, natural substrates) increases the expression of NeuroD1 mRNA, which is a gene associated with learning, memory, and the ability to forage (Rodewald et al., 2011; Cámara-Ruiz et al., 2019). Fishes reared in an enriched environment are also less likely to engage in risky behaviors that increase the chances of contact with predators (Roberts et al., 2011). Changes in feed types also modify the gastrointestinal microbiome, and this can influence the digestion, assimilation, and utilization of nutritional components. Introducing natural feed earlier (under hatchery conditions) can facilitate this process and counteract the stress linked with changing diets (Yang et al., 2020). One effect of these actions could be increased stocking material survival following release (Hyvärinen and Rodewald, 2013).

Research on modulating the foraging behavior of hatchery-reared stocking material analyzes primarily rearing indicators and fish behavior (Czerniawski et al., 2015; Krepski and Czerniawski, 2019). Changing diets and rearing conditions can be stress factors for fish that can affect physiological condition (Sopinka et al., 2016). Conclusive methods for assessing fish health condition include analyzing hematological, biochemical, and immune indicators (Collins et al., 2016). Research on the influence of given rearing conditions on hematological and plasma biochemical parameters provides good information on fish body function, nutritional status, and the occurrence of stress (e.g., cortisol, glucose, lactate) (Sopinka et al., 2016; Fazio, 2019; Ciji and Akhtar, 2021; Witeska et al., 2021). Few studies to date have focused on fish hematological indicators, especially those of physiological responses to procedures applied in aquaculture (e.g., changing feed, sorting, transport, etc.). To the best of the authors' knowledge, the influence of changes in diet (e.g., from formulated to natural feeds) on fish health in the period preceding stocking have not yet been analyzed. Understanding these reactions and drawing concrete conclusions concerning them will be helpful for developing procedures that improve the vitality of stocking material prior to its release into open waters.

The aim of the current research was to determine the effects of changing diets (from formulated to natural feed, i.e., prey fish) and the length of time the diets were applied (two or four weeks) on the condition and health (hematological, biochemical, and immunity indicators) of juvenile sea trout reared in aquaculture facilities.

## Material and methods

### Fish origin

Fertilized sea trout eggs were obtained in late October and early November from fish in the broodstock maintained at the Department of Salmonid Research, Inland Fisheries Institute in Olsztyn (DSR IFI Olsztyn). Among the broodstocks at the DSR IFI Olsztyn is a sea trout broodstock that was built using the Vistula sea trout population (Bernaś and Wąs-Barcz, 2020). All of the tanks in which the fish are kept were supplied with water from the Radunia River (Kashubian Lake District, northern Poland). Rearing procedures for sea trout were applied that were developed at DSR IFI Olsztyn over the course of long-term research on the culture of this species (S. Dobosz, unpublished data). After one year of rearing, the sea trout were sorted for the first time. Fish that had attained a body weight of approximately 30 g (± 5 g) were left in the same tanks. For the two months prior to the experiment, the fish were fed T-1P Via Ultra feed (Skretting®, Stavanger, Norway) with a granule size of 3 mm containing 45% crude protein, 24% crude fat, 15.5% nitrogen free extract (NFE), 2% fiber, 6% crude ash, and 21 MJ/kg total energy. The fish were fed manually six times daily between 08:00 and 16:00. One month prior to the experiment feed was delivered with a band feeder (FIAP, Fishtechnik GmbH, Ursensollen, Germany) also for 8 h/d (08:00–16:00). The tanks with the larval and juvenile sea trout were supplied with surface water (Radunia River) of the following parameters: pH approximately 7.0; dissolved oxygen saturation 81–95%; temperature from 0.1°C in winter to 21.7°C in summer.

### Material, rearing conditions, feeding, and diet composition

A total of 480 fish aged 16 months after hatching were used. The mean ± SD fork length (FL) of the fish was approximately 18.7±1.3 cm, standard length (SL)

was 17.4±1.3 cm, and body weight (BW) was 73±16.6 g. The fish were stocked into tanks of the same volume of 1.14 $m^3$ (1.95×1.95×0.3 m; L×W×H). A total of 12 rearing tanks were stocked with 40 fish each. Throughout the experiment, which ran for four weeks, the fish were held in optimum conditions recommended for rearing *Salmo trutta*. Measurements of water temperature (± 0.1°C) and oxygen concentration (± 0.01 mg $O_2$/L) at the rearing tank inflows and outflows were taken daily. The other water parameters such as total ammonia nitrogen (TAN = $NH_4^+$-N + $NH_3$-N) (± 0.01 mg TAN/L), nitrite $NO_2$-N (± 0.01 mg $NO_2$-N/L), and water pH (± 0.01) were measured at the rearing tank outflows every seven days. Standard colorimetric methods were used (APHA, 1999). The mean ± SD water temperature was 11.7±0.2°C. Dissolved oxygen concentration at the rearing tank outflows did not fall below 9.03 mg $O_2$/L (saturation 85.1%). Dissolved oxygen saturation at the inflows was maintained within a range of 91–98%. The concentrations of TAN and $NO_2$-N at the outflows did not exceed 0.089 mg TAN/L or 0.009 mg $NO_2$-N/L. Water pH (measured at the outflows) was within the range of 7.78–7.86. Natural lighting and a natural photoperiod were applied in the rearing hall (the walls and roof of which are constructed partially of reinforced glass). The rearing tanks were covered with plastic netting with a mesh size of 15×15 mm.

Four diet groups were created, each in three replicates (4 × 3 tanks). The diet of the sea trout in group A was not changed, and they were fed Skretting® commercial feed (3 mm granules). The fish in group B were fed BioMar EFICO Enviro® commercial feed (Biomar, Aarhus, Denmark) (3 mm granules; diet B). Groups B/N and N received natural feed (diet N – prey fish), which was rainbow trout (*Oncorhynchus mykiss*) juveniles (aged 2.5 months; SL 6.61±0.56 cm; FL 7.23±0.60 cm; BW 4.14±0.92 g (mean ± SD); spring spawning). For the initial two weeks, the fish from group B/N were fed mixed feed (B + N). Half of the feed ration was BioMar feed and half was rainbow trout juveniles. In the subsequent two weeks, the fish were fed diet N exclusively. Group N was fed natural feed (diet N) throughout the feeding test of four weeks. Commercial feed was delivered by automated band feeders (FIAP, Fishtechnik GmbH, Ursensollen, Germany) according to D-journal Freshwater Farm Software® (From and Rasmussen, 1984). The ration of formulated feed was approximately 1% of the stock biomass/d. This program calculates the daily feed ration based on water temperature, feed energy values, fish stocking density, and fish body weight. The program was developed for rainbow trout. It was determined that the optimum feed ration for *Salmo trutta* is 80% of the estimated ration for rainbow trout (S. Dobosz, personal communication). It was also assumed that the feed conversion ratio (FCR) of formulated feed for juvenile sea trout was 1.0, and for prey fish it was 3.0. Natural feed was delivered to groups B/N and N once daily at 08:00 after the rearing tanks were cleaned from 07:00 to 08:00. The amounts of feed consumed, fish mortality, and fish behavior were also monitored for four weeks.

Table 1. Proximate composition (% wet weight) and fatty acids profiles (% total fatty acids) of the diets tested

| Specification | Tested diets | | |
|---|---|---|---|
| | diet A | diet B | prey fish |
| Proximate composition | | | |
| Crude protein | 43.45 | 44.37 | 17.14 |
| Crude fat | 14.52 | 22.38 | 1.54 |
| Nitrogen free extract (NFE)[1] | 29.02 | 22.65 | - |
| Crude ash | 6.14 | 5.20 | 1.05 |
| Water | 6.87 | 5.40 | 80.27 |
| Gross energy (MJ/kg feed)[2] | 21.02 | 23.23 | 4.71 |
| Fatty acids composition | | | |
| C12:0 | 0.53 | 0.10 | - |
| C14:0 | 2.14 | 2.59 | 3.90 |
| C15:0 | 0.15 | 0.20 | 0.27 |
| C16:0 | 17.09 | 11.25 | 13.36 |
| C17:0 | 0.21 | 0.16 | 0.18 |
| C18:0 | 5.56 | 3.08 | 2.69 |
| C20:0 | 0.49 | 0.45 | 0.28 |
| C22:0 | 0.82 | 0.27 | 0.21 |
| SFA | 26.98 | 18.10 | 20.88 |
| C14:1 | 0.10 | 0.02 | 0.02 |
| C16:1 | 4.24 | 2.63 | 4.02 |
| C17:1 | 0.31 | 0.27 | 0.34 |
| C18:1 cis9 | 33.12 | 43.17 | 34.30 |
| C18:1 cis11 | 2.23 | 2.60 | 2.70 |
| C20:1 n-9 | 0.86 | 3.41 | 5.38 |
| C20:1 n-7 | 0.05 | 0.12 | 4.93 |
| C22:1 n-11 | 0.47 | 3.62 | - |
| C22:1 n-9 | 0.10 | 0.36 | 0.51 |
| MUFA | 41.48 | 56.21 | 52.19 |
| C18:2 n-6 | 16.41 | 13.91 | 10.16 |
| C18:3 n-6 | 0.23 | 0.16 | 0.26 |
| C18:3 n-3 | 10.66 | 5.88 | 3.41 |
| C18:4 n-3 | 0.39 | 0.65 | 0.92 |
| C20:2 n-6 | 0.11 | 0.37 | 0.53 |
| C20:3 n-6 | 0.00 | 0.00 | 0.21 |
| C20:4 n-6 | 0.39 | 0.17 | 0.37 |
| C20:3 n-3 | 0.00 | 0.17 | 0.18 |
| C20:4 n-3 | 0.00 | 0.25 | 0.59 |
| C20:5 n-3 | 2.03 | 1.72 | 2.22 |
| C22:5 n-6 | - | - | 0.13 |
| C22:5 n-3 | 0.24 | 0.29 | 0.83 |
| C22:6 n-3 | 1.08 | 2.11 | 7.14 |
| PUFA | 31.53 | 25.69 | 26.93 |
| n-3 PUFA | 14.40 | 11.07 | 15.28 |
| n-6 PUFA | 17.13 | 14.62 | 11.65 |
| n-3 PUFA/n-6 PUFA | 0.84 | 0.76 | 1.31 |

[1]NFE = 100 - (crude protein + crude fat + crude ash + water); [2]Gross energy calculated from the proximate composition using the following energy conversion factors: 24 kJ/g proteins; 39 kJ/g fat; 17 kJ/g NFE (Jobling, 1994).

SFA – saturated fatty acids, MUFA – monounsaturated fatty acids, PUFA – polyunsaturated fatty acids, n-3 PUFA contain: 18:3 n-3; 18:4 n-3; 20:3 n-3; 20:4 n-3; 20:5 n-3; 22:5 n-3; 22:6 n-3, n-6 PUFA contain: 18:2 n-6; 18:3 n-6; 20:2 n-6; 20:3 n-6; 20:4 n-6, 22:5 n-6.

Component composition (manufacturer data):

diet A – fish meal, poultry meal, soya concentrate, hydrolyzed feather protein meal, blood meal, wheat gluten, wheat, fish oil, poultry fat, linseed oil, rapeseed oil;

diet B – fish meal, poultry meal, soya concentrate, blood meal, fish oil, rapeseed oil, horse beans, wheat, hydrolyzed feather protein meal, sunflower cake, guar protein, wheat gluten, monosodium phosphate, monocalcium phosphate, yeast.

The proximate composition of the experimental feeds (A, B, N) was analyzed according to AOAC (2007) procedures: crude protein was determined with the Kjeldahl method (Kjel-Foss Automatic 16210 analyzer; AISN Foss Electric, Hillerød, Denmark); crude lipid with the Soxhlet method (extraction with ethyl ether for 12 h); crude ash by incinerating a dried sample at a temperature of 550°C for 12 h (furnace by Linn High Therm GmbH; Eschenfelden, Germany). The gross energy of the feed was calculated based on proximate composition using the following energy conversion factors: 39 kJ/g lipids; 24 kJ/g protein; 17 kJ/g carbohydrates (Jobling, 1994). Nitrogen free extract (NFE) was calculated based on the difference (100 – (water + crude lipids + crude protein + crude ash)) (Table 1).

The fatty acid composition of the fats contained in the diets tested (A, B, N) was analyzed after the muscle lipids were cold extracted (Table 1). Fatty acid methylation was conducted with a mixture of chloroform:methanol:sulfuric acid (100:100:1). Chromatographic separation was performed on an Agilent Technologies 6890 N gas chromatograph with a flame ionization detector (FID) and a 30 m capillary column with an internal diameter of 0.32 mm. The liquid phase was Supelcowax 10 with a film thickness 0.25 µm. The separation conditions were as follows: helium carrier gas flow of 1 mL/min; detector temperature – 250°C; injector temperature – 225°C; column temperatures – 180°C. The detector signals were recorded using a Philips recorder on a scale of 1 mV at a belt speed of 10 mm/min. Individual acids were identified by comparing retention times with standards from Supelco (Bellefonte, PA, USA; details in Zakęś et al., 2010).

**Experimental procedures and sampling**

At the beginning of the experiment (day 0; d0), three sea trout samples (30 specimens each) were collected at random. Fork length (FL ± 1 mm), standard length (SL ± 1 mm), and body weight (BW ± 0.01 g) were measured. These fish were the initial sample. Subsequently, individual fish were measured after 14 and 28 days of rearing. These data were used to calculate the following rearing indices: daily growth rate – DGR (g/d) = $(BW_2 – BW_1) / t$; specific growth rate – SGR (%/d) = $100 \times (\ln BW_2 – \ln BW_1) / t$; condition factor – CF = $100 \times (BW / SL^3)$; feed conversion ratio – FCR = TFS / (FB – IB), where: $BW_1$ – initial fish body weight (g), $BW_2$ – final fish body weight (g), t – rearing time (days), SL – standard length (cm), FB – final stocking biomass (g), IB – initial stocking biomass (g), TFS – total feed supplied (g). Mortality in the tanks was monitored daily.

**Hematological and serum biochemical analysis**

Blood for testing (approximately 1 mL) was drawn from the caudal vein before the beginning of the experiment and also after 14 and 28 days (d0, d14, and d28) from seven fish sampled at random from each group (two or three fish from each tank/replicate). Blood was drawn using heparinized syringes (Smiths Medical International ASD, Inc., Minnesota, USA). All the fish were handled (for measurements and drawing blood) under general anesthesia induced with an aqueous solution of methanesulphonate (MS-222) (Sigma-Aldrich Co., Missouri, USA) at a dose of 100 mg/L. The following hematological parameters were determined in the material collected: white blood cell (WBC) count; red blood cell (RBC) count; hemoglobin (HGB); hematocrit (HCT). Red blood cell indices were also calculated, as follows: mean corpuscular volume (MCV); mean corpuscular hemoglobin (MCH); mean corpuscular hemoglobin concentration (MCHC). The remaining part of the blood samples were centrifuged at 4,000 rpm for 3 min (Fresco 17, Thermo Scientific, Massachusetts, USA). The following plasma biochemical parameters were determined: cortisol (CORT); glucose (GLU); triglycerides (TG); cholesterol (CHOL); total protein (TP); albumin (ALB); globulin (GLB); total bilirubin (BIL-T); lactate (LACT); ammonia (AMON); alanine aminotransferase (ALT); aspartate aminotransferase (AST); alkaline phosphatase (ALP); potassium ions ($K^+$); sodium ions ($Na^+$); chloride ions ($Cl^-$). The hematology tests were performed with a BC-2800 VET semi-automatic hematology analyzer (Mindray, Shenzhen, China) (Fazio, 2019; Minaz et al., 2022). Analyzer readings were calibrated for several fish species, including sea trout, based on the results of hematological tests performed with traditional methods (Dacie and Lewis, 2001). Stamar (Dąbrowa Górnicza, Poland), the device distributor, performed the calibrations. The biochemical blood plasma tests were performed with a BS-120 automatic biochemistry analyzer (Mindray, Shenzhen, China). Dedicated kits were used to determine the values of the different parameters (Stamar, Dąbrowa Górnicza, Poland). Cortisol concentrations were determined with ELISA (Cayman Chemical Company, Michigan, USA). The hormone was extracted from the plasma with ethyl ether according to the method in Hermelink et al. (2011). The analyses were conducted in 96-well plates onto which the following were applied: EIA test buffer; cortisol standards; the samples tested (each in two replicates). The optimum dilution of the samples analyzed was determined previously through a series of tests. Following incubation and rinsing the wells, the samples were read at a wavelength of 412 nm. According to the manufacturer's (Cayman Chemical Company, Michigan, USA) instructions, the cortisol levels of the samples were calculated using the standard curve.

**Measurements of phagocytic activity**

The level of nonspecific immunity in the sea trout was determined from leukogram analysis and assessing the activity of the enzyme myeloperoxidase ($A_{MPO}$) present in granulocyte and monocyte granules (Das and Sahoo, 2014). The procedure and timing (d0, d14, and d28) of blood sampling and the sample size of each group (n = 7) were in accordance with standards used to sample blood for the hematological and biochemical determinations described above.

Phagocytic activity measurements:

– zymosan preparation: 600 mg of zymosan (Sigma-Aldrich, USA) was mixed with 15 mL Hanks Balanced Salt Solution (HBSS) and heated for 20 min in a water bath at 70°C. After cooling, it was centrifuged (5 min in 600 *g*) and rinsed twice in 30 mL of HBSS and then diluted in 30 mL HBSS to reach a concentration of 20 mg/mL,
– phagocytic activity measurement: for each fish, 200 µL aliquots of blood were placed in 2 wells of a microplate (NUN96ft, Thermo Fisher Scientific) and 15 µL of zymosan solution was added to each well. Two smears of blood from each well were done after 25 min incubation in the chamber (MaxQ 4450, Thermo Scientific) at 23°C with continuous stirring. Smears were stained with the standard Giemsa procedure and then analyzed under a light microscope. For each fish, at least 200 leukocytes were determined to the type and the relative number of phagocytes (rPhC) and the percentage of active phagocytes (rAPhC) were determined with the following equations (Nikoskelainen et al., 2004):

$$rPhC\ (\%) = 100 \times [(\text{neutrophil count} + \text{monocyte count}) / \text{leukocyte count}],$$
$$rAPhC\ (\%) = 100 \times (\text{count of phagocytes with zymosan inside} / \text{total phagocyte count}).$$

The number of phagocytes (PhC), the number of active phagocytes (APhC), and the phagocytic index (PhI) were calculated with the following equations (Santulli-Marott et al., 2015):

$$PhC\ (10^3/\mu L) = rPhC \times WBC,$$
$$APhC\ (10^3/\mu L) = rAPhC \times PhC,$$
$$PhI = ZP / APhC,$$

where: *WBC* – white blood cell count, *ZP* – zymosan particle counts in phagocytes.

Myeloperoxidase activity ($A_{MPO}$) measurements: two smears were prepared from each blood sample. These smears were subjected to multi-stage cytochemical staining with a Peroxidase Activity Assay Kit (Sigma Aldrich, USA) according to the manufacturer's instructions. The key stage of the procedure was incubation in the presence of diaminobenzidine (DAB) and hydrogen peroxide. In the presence of myeloperoxidase, DAB oxidizes to a brown color and then changes to gray-black in the next steps of cytochemical staining. Thus, the granules containing MPO in the macrophages are colored gray to black. Depending on the intensity of the color and the size and extent of the granules, four levels of $A_{MPO}$ were distinguished from inactive (grade 0) to strongly active (grade 3).

Cidal ability (CA) was calculated as follows:

$$CA = A_{MPO} \times APhC.$$

**Statistical analysis**

Statistical analysis was performed with Statistica 13.3 (StatSoft, Inc., USA). Compliance of variable distributions with the normal distribution was tested with the Shapiro-Wilk test. Data normality was checked with Levene's test and the Brown-Forsythe test. For variables that met the assumptions of data normality and homogeneity, the one-way ANOVA procedure was used to analyze differences among means (growth parameters [FL, SL, BW, F], $A_{MPO}$, red blood cell indices, glucose, cholesterol, total protein, albumin, globulin, ALT, AST, ALP, $K^+$, $Na^+$, $Cl^-$). For other parameters, non-parametric Kruskal-Wallis ANOVA and Dunn's test were used (white blood cell differential count, APhC, PhI, CA, BIL-T, LACT, WBC, CORT, rearing indices [SGR, DGR, FCR]). Two-factor analysis of variance was used to detect the influence of potential interactions between feed (factor D) and feeding time (factor T) on the variables studied (phagocytic parameters; WBC; red blood cell indices; biochemical and stress indices; growth parameters). Differences were significant at $P \leq 0.05$.

## Results

**Growth performance**

No fish mortality was noted during the experiment. Two-factor ANOVA analysis did not indicate the diets tested (D) had any significant influence on increased body growth (FL or SL) or body weight (BW), but rearing time (T) was significant. No statistically significant interactions among the factors tested were found (D×T; $P>0.05$; Table 2). The diets ($F = 6.16$; $P<0.01$) and feeding period ($F = 59.20$; $P<0.001$) significantly influenced fish condition (CF). Fish from groups A and B that were fed formulated feed exclusively had higher CF values (Table 2). No significant differences were noted with regard to DGR or SGR among the groups ($P>0.05$). After two weeks of the test (days 1–14), the FCR values in the groups fed formulated feed exclusively were significantly lower than in the groups fed prey fish (group N) and mixed feed (formulated feed B and prey fish – group B/N). Differences in FCR values between groups N and B/N were also significant ($P \leq 0.05$; Table 3). In the subsequent two weeks of rearing (weeks three and four; days 15–28), the FCR values in group B/N were close to those calculated for group N (Table 3). The FCR values for the entire four-week test were as follows: group A = group B < group B/N < group N.

**Hematological indicators**

Factors D and T did not influence WBC counts ($P>0.05$), but the diets had a significant influence on the values of RBC ($F = 26.88$; $P<0.001$), HGB ($F = 29.23$; $P<0.001$), and HCT ($F = 38.09$; $P<0.001$). However, decreased values of these indicators were noted after two weeks of the test, and after four weeks of the test they did not differ significantly from the values of the initial fish sample (Table 4). The diets also had a significant influence on the RBC indicators of MCH ($F = 16.7$; $P<0.001$) and MCHC ($F = 13.2$; $P<0.001$). After two weeks of the test the values of MCH and MCHC increased significantly in group N, and after four weeks they decreased and did not differ from those in the other groups (Table 4).

Table 2. Results of ANOVA and two-factor ANOVA analyses of the growth parameters of juvenile sea trout fed different feeds

| Parameter | Time of feeding (days) | Feeding groups | | | | Factor effect significance (F statistic and P value) | | | Model fit goodness | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | group A | group B | group N | group B/N | feed (D) | feeding time (T) | D × T | $R^2$ | F statistic and P-value |
| FL | 0 | 18.7±1.3 | | | | F = 0.06 P = 0.98 | F = 11.4 P<0.001 | F = 0.08 P = 1.00 | 0.26 | F = 2.13 P =0.059 |
| | 14 | 20.0±0.6 | 20.0±0.1 | 20.1±0.6 | 19.9±0.3 | | | | | |
| | 28 | 20.3±0.05 | 20.4±0.3 | 20.1±0.3 | 20.0±0.2 | | | | | |
| SL | 0 | 17.4b±1.3 | | | | F = 0.00 P = 1.00 | F = 14.04 P<0.001 | F = 0.07 P = 1.00 | 0.33 | F = 2.59 P<0.05 |
| | 14 | 18.6 ab±0.7 | 18.6 ab±0.1 | 18.8 ab±0.6 | 18.8 ab±0.6 | | | | | |
| | 28 | 19.1 a±0.1 | 19.1 a±0.3 | 18.9 ab±0.3 | 18.8 ab±0.2 | | | | | |
| BW | 0 | 73.4 b±16.6 | | | | F = 0.34 P = 0.80 | F = 26.88 P<0.001 | F = 0.22 P =0.97 | 0.56 | F = 5.10 P<0.001 |
| | 14 | 101.0 a±13.6 | 100.0 a±3.0 | 99.2 a±13.3 | 98.6 ab±4.8 | | | | | |
| | 28 | 108.0 a±2.4 | 112.0 a±4.4 | 101.0 a±4.7 | 101.0 a±0.8 | | | | | |
| CF | 0 | 1.37 c±0.02 | | | | F = 6.16 P<0.01 | F = 59.20 P<0.001 | F = 2.43 P = 0.06 | 0.80 | F = 13.77 P<0.001 |
| | 14 | 1.54 ab±0.05 | 1.53 ab±0.02 | 1.47 bc±0.06 | 1.50 ab±0.04 | | | | | |
| | 28 | 1.54 ab±0.01 | 1.60 a±0.07 | 1.46 bc±0.01 | 1.48 bc±0.30 | | | | | |

Mean values ± SD (n = 3). Parameter values with different letter differ significantly (one way ANOVA; P≤0.05). A P value lower than 0.05 indicated the factor had a significant effect. Group A – formulated feed (Skretting®); group B – formulated feed (BioMar®); group B/N – mixed feed (BioMar® and prey-fish); group N – prey fish. Details in Material and methods. FL – fork length (cm); SL – standard length (cm); BW – body weight (g); CF – condition factor (-).

Table 3. Specific and daily growth rates and feed conversion ratio of juvenile sea trout fed different feeds

| Group | Time of feeding (days) | DGR | SGR | FCR |
|---|---|---|---|---|
| A | 1–14 | 1.32 a±0.14 | 1.31 a±0.14 | 0.81 c±0.10 |
| B | | 1.37 a±0.20 | 1.36 a±0.20 | 0.79 c±0.12 |
| N | | 1.13 a±0.04 | 1.12 a±0.04 | 2.80 a±0.11 |
| B/N | | 1.28 a±0.02 | 1.27 a±0.02 | 1.68 b±0.02 |
| A | 15–28 | 0.78 a±0.47 | 0.78 a±0.46 | 1.17 b±0.24 |
| B | | 0.88 a±0.26 | 0.88 a±0.25 | 1.10 b±0.41 |
| N | | 0.46 a±0.07 | 0.46 a±0.07 | 3.71 a±0.53 |
| B/N | | 0.64 a±0.21 | 0.63 a±0.20 | 3.61 a±0.19 |
| A | 1–28 | 1.07 a±0.29 | 1.07 a±0.29 | 0.94 c±0.14 |
| B | | 1.14 a±0.04 | 1.14 a±0.04 | 0.89 c±0.01 |
| N | | 0.82 a±0.04 | 0.82 a±0.04 | 3.15 b±0.10 |
| B/N | | 0.98 a±0.10 | 0.98 a±0.10 | 2.30 a±0.03 |

Mean values ± SD (n = 3). Values in columns with different letter in given periods of time differ significantly statistically (P≤0.05). A – formulated feed (Skretting®); B – formulated feed (BioMar®); B/N – mixed feed (BioMar® and prey-fish); N – prey fish. Details in Material and methods. DGR – daily growth rate (g/d); SGR – specific growth rate (%/d); FCR – feed conversion ratio (–).

**Proportion of different blood leukocytes (leukogram) and phagocytic indices**

After two weeks of the test, the number of small lymphocytes (S lymphocytes) and total lymphocytes in group B were significantly higher than those in the initial fish sample (P≤0.05). After four weeks, these differences were noted in groups B and B/N (in comparison to the initial fish sample) (P≤0.05; Table 5). Significantly lower numbers of neutrophils MN and total neutrophils were confirmed in groups B and B/N after two weeks of the test (P≤0.05). After four weeks, these values did not differ from those estimated for initial fish sample at the beginning of the experiment. No significant differences were noted in the values of the eosinophils or monocytes (P>0.05; Table 5).

Table 4. Results of ANOVA and two-factor ANOVA analyses of the hematological parameters of juvenile sea trout fed different feeds

| Parameter | Time of feeding (days) | Feeding groups | | | | Factor effect significance (F statistic and P value) | | | Model fit goodness | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | group A | group B | group N | group B/N | feed (D) | feeding time (T) | D × T | $R^2$ | F statistic and P-value |
| WBC | 0 | 262±51 | | | | F = 1.063 | F = 0.386 | F = 0.161 | 0.09 | F = 0.375 |
| | 14 | 267±8 | 277±7 | 267±10 | 271±9 | P = 0.35 | P = 0.76 | P = 0.99 | | P = 0.96 |
| | 28 | 270±11 | 282±7 | 265±12 | 280±8 | | | | | |
| RBC | 0 | 0.61 a±0.08 | | | | F = 26.88 | F = 0.224 | F = 0.220 | 0.35 | F = 5.07 |
| | 14 | 0.48 b±0.06 | 0.50 ab±0.03 | 0.45 b±0.06 | 0.48 b±0.06 | P<0.001 | P = 0.88 | P = 0.97 | | P<0.001 |
| | 28 | 0.58 a±0.07 | 0.57 ab±0.05 | 0.58 a±0.09 | 0.60 a±0.10 | | | | | |
| HGB | 0 | 54.1 a±3.7 | | | | F = 29.23 | F = 2.650 | F = 0.96 | 0.42 | F = 6.56 |
| | 14 | 45.3 c±3.4 | 49.2 ac±2.8 | 46.4 bc±4.0 | 46.4 bc±3.5 | P<0.001 | P = 0.06 | P = 0.46 | | P<0.001 |
| | 28 | 48.0 ac±5.0 | 51.8 ab±2.5 | 45.8 bc±5.9 | 48.0 ac±1.4 | | | | | |
| HCT | 0 | 17.7 a±1.5 | | | | F = 38.09 | F = 0.254 | F = 0.189 | 0.45 | F = 7.10 |
| | 14 | 13.5 bc±1.9 | 13.8 bc±1.0 | 12.6 c±1.9 | 13.4 bc±1.9 | P<0.001 | P = 0.86 | P = 0.98 | | P<0.001 |
| | 28 | 16.5 ab±2.2 | 16.1 ac±1.6 | 16.1 ac±2.5 | 16.4 ab±2.8 | | | | | |
| MCV | 0 | 206±5 | | | | F = 1.5 | F = 2.7 | F = 1.2 | 0.08 | F = 1.66 |
| | 14 | 209±6 | 207±3 | 207±5 | 207±6 | P = 0.24 | P = 0.05 | P = 0.30 | | P = 0.10 |
| | 28 | 212±1 | 208±6 | 207±4 | 203±3 | | | | | |
| MCH | 0 | 146 ab±12 | | | | F = 16.7 | F = 0.65 | F = 1.0 | 0.27 | F = 3.76 |
| | 14 | 156 ab±18 | 163 ac±8 | 171 a±27 | 160 ab±19 | P<0.001 | P = 0.58 | P = 0.43 | | P<0.001 |
| | 28 | 137 bc±16 | 149 ab±14 | 131 c±14 | 134 bc±22 | | | | | |
| MCHC | 0 | 709 ab±62 | | | | F = 13.2 | F = 0.62 | F = 0.78 | 0.21 | F = 3.00 |
| | 14 | 751 ab±108 | 789 ab±53 | 829 a±148 | 778 ab±108 | P<0.001 | P = 0.60 | P = 0.59 | | P<0.01 |
| | 28 | 649 b±74 | 720 ab±83 | 636 b±73 | 664 b±111 | | | | | |

Mean values ± SD (n = 7). Parameter values with different letter differ significantly (one way ANOVA; P≤0.05). A P value lower than 0.05 indicated the factor had a significant effect. Group A – formulated feed (Skretting®); group B – formulated feed (BioMar®); group B/N – mixed feed (BioMar® and prey-fish); group N – prey fish. Details in Material and methods. WBC – white blood cells ($10^3$/μL); RBC – red blood cells ($10^6$/μL); HGB – hemoglobin (g/L); HCT – hematocrit (%); MCV – mean corpuscular volume (fl); MCH – mean corpuscular hemoglobin (pg); MCHC – mean corpuscular hemoglobin concentration (g/L).

Table 5. White blood cell differential count (percentage of total white blood cells in blood smears; upper values) and absolute count ($10^3$/μL; lower values) of juvenile sea trout fed different feeds

| Time of feeding (days) | Group | Lymphocytes S | Lymphocytes L | Total lymphocytes | Neutrophils JN | Neutrophils MN | Total neutrophils | Eosinophils | Monocytes |
|---|---|---|---|---|---|---|---|---|---|
| 0 | Initial | 85.9±2.4 | 7.5±2.1 | 93.4±2.6 | 1.7±0.8 | 4.6±2.4 | 6.3±2.7 | 0.1±0.1 | 0.2±0.4 |
| | | 225.4 bc±6.3 | 19.8±5.7 | 245.2 b±6.8 | 4.5±2.0 | 12.1 a±6.2 | 16.5 a±7.1 | 0.1±0.4 | 0.6±0.9 |
| 14 | A | 91.6±3.6 | 3.8±1.8 | 95.4±2.6 | 1.5±0.5 | 2.8±2.6 | 4.3±2.6 | 0.0 | 0.3±0.3 |
| | | 244.3 ab±9.6 | 10.1±4.8 | 254.4 b±7.1 | 3.9±1.4 | 7.5 ab±7.0 | 11.4 ab±6.9 | | 0.8±0.9 |
| | B | 91.9±2.6 | 5.8±2.8 | 97.7±0.8 | 1.2±0.4 | 0.01±0.002 | 2.0±0.6 | 0.1±0.2 | 0.2±0.2 |
| | | 254.4 aa±9.6 | 16,0±7.7 | 270.3 a±2.1 | 3.3±1.1 | 2.38 b±0.6 | 5.6 b±1.6 | 0.2±0.5 | 0.6±0.7 |
| | N | 91.9±3.2 | 5.2±3.2 | 97.1±0.3 | 1.5±0.4 | 1.0±0.4 | 2.5±0.4 | 0.2±0.3 | 0.2±0.2 |
| | | 245.2 a±8.5 | 13.8±8.6 | 259.0 ab±0.9 | 4.0±1.0 | 2.7 ab±1.0 | 6.7 ab±1.0 | 0.5±0.7 | 0.5±0.6 |
| | B/N | 92.6±3.9 | 5.0±3.2 | 97.7±0.8 | 1.2±0.4 | 0.8±0.3 | 2.0±0.5 | 0.1±0.2 | 0.2±0.4 |
| | | 244.0 ab±10.3 | 13.2±8.4 | 257.0 b±2.2 | 3.2±1.1 | 2.2 b±0.9 | 5.4 b±1.3 | 0.1±0.5 | 0. 5±1.0 |
| 28 | A | 90.3±3.4 | 6.4±3.2 | 96.6±1.0 | 1.2±0.5 | 1.8±0.5 | 3.0±0.7 | 0.1±0.2 | 0.3±0.4 |
| | | 244.1 ab±10.2 | 17.3±8.6 | 261.3 ab±2.7 | 3.2±1.4 | 4.9 ab±1.3 | 8.1 ab±2.0 | 0.3±0.5 | 0.7±1.0 |
| | B | 91.3±2.4 | 4.7±3.0 | 96.0±1.0 | 1.2±0.4 | 2.4±1.4 | 3.7±1.1 | 0.1±0.2 | 0.3±0.4 |
| | | 257.4 a±6.8 | 13.2±7.8 | 270.6 a±2.8 | 3.5±1.2 | 6.9 ab±4.0 | 10.4 ab±3.1 | 0.1±0.5 | 0.7±1.0 |
| | N | 88.1±5.3 | 8.4±4.5 | 96.5±1.7 | 1.3±0.6 | 1.9±1.4 | 3.2±1.4 | 0.1±0.1 | 0.3±0.4 |
| | | 233.2 b±14.0 | 22.1±11.9 | 255.4 b±4.6 | 3.4±1.6 | 5.0 ab±3.6 | 8.5 ab±3.8 | 0.1±0.3 | 0.7±1.1 |
| | B/N | 92.0±2.6 | 4.3±3.7 | 96.3±1.4 | 1.3±0.5 | 2.0±0.9 | 3.3±1.2 | 0.1±0.2 | 0.2±0.3 |
| | | 258.0 a±7.4 | 12.0±10.3 | 270.0 a±3.8 | 3.7±1.5 | 5.6 ab±2.6 | 9.3 ab±3.4 | 0.4±0.6 | 0.7±0.9 |

Mean values ± SD (n = 7). Values in columns with different letter differ significantly (P≤0.05). A – formulated feed (Skretting®); B – formulated feed (BioMar®); B/N – mixed feed (BioMar® and prey-fish); N – prey fish. Details in Material and methods. Lymphocytes S – small lymphocytes; Lymphocytes L – large lymphocytes; Neutrophils JN – juvenile neutrophils; Neutrophils MN – mature neutrophils.

Two-factor ANOVA revealed that the percentage of active phagocytic cells (APhC; F = 49.7; P<0.001), the phagocytic index (PhI; F = 30.0; P<0.001), and cidal ability (CA; F = 37.64; P<0.001) were significantly determined by the duration for which a given diet was provided. After two weeks, the indices of APhC and CA were significantly lower in groups B, N, and B/N (this change was not noted in group A, in which the diet was not changed), while after four weeks these reverted to the values noted in the initial fish sample (IS; Figures 1, 2, 3). $A_{MPO}$ activity was significantly associated with both the influence of the diets (F = 22.21; P<0.001) and the duration of their provision (F = 361.41; P<0.001). Interactions between factors D and T were also significant (F = 40.59; P<0.001). A significant decrease in $A_{MPO}$ values was noted after two weeks of the test in groups B, N, and B/N, while after four weeks the value of this indicator did not differ from that confirmed in initial fish sample. Only in group N was the $A_{MPO}$ value higher (P<0.01). In group A, the value of this indicator remained at a stable level throughout the rearing period (Figure 4).

**Biochemical indicators of nutritional status**

The diets significantly influenced triglycerides (F = 13.3; P<0.001). Cholesterol levels were significantly determined by both factors D (F = 54.6; P<0.001) and T (F = 28.1; P<0.001). Interactions between D and T were also significant (F = 8.5; P<0.001). After two weeks, CHOL values increased significantly in group N and in groups N and B/N after four weeks (Table 6). Diet significantly influenced the levels of total protein (F = 57.7; P<0.001), globulin (F = 23.0; P<0.001), and total bilirubin (F = 6.0; P<0.01). The values of the liver indicators of ALT and AST were similar throughout the test (P>0.05; Table 6).

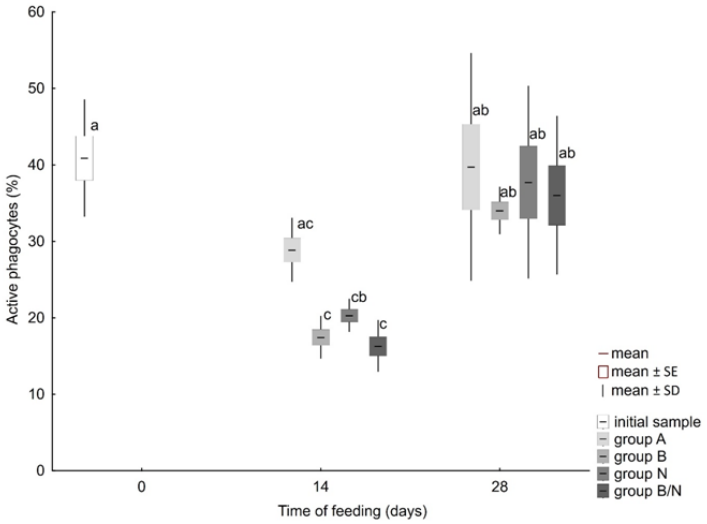



Figure 1. Percentage of phagocytes engulfing zymosan in the blood of juvenile sea trout fed formulated feeds (groups A and B), the mixed diet (feed B and prey fish; group B/N), prey fish (group N) confirmed after 0, 14, and 28 days of the trial. Details in Material and methods. Values with different letter indexes differed significantly at P≤0.01

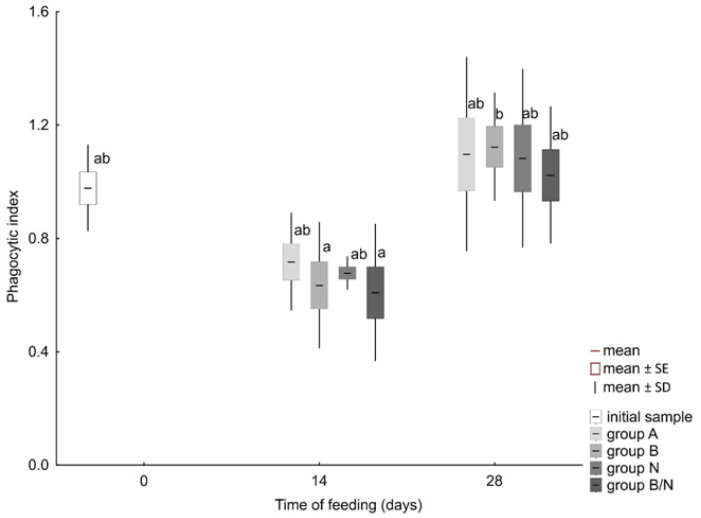



Figure 2. Phagocytic index (mean number of zymosan particles engulfed by a single phagocytic cell) of juvenile sea trout fed formulated feeds (groups A and B), the mixed diet (feed B and prey-fish; group B/N), prey fish (group N) confirmed after 0, 14, and 28 days of the trial. Details in Material and methods. Values with different letter indexes differed significantly at P≤0.05

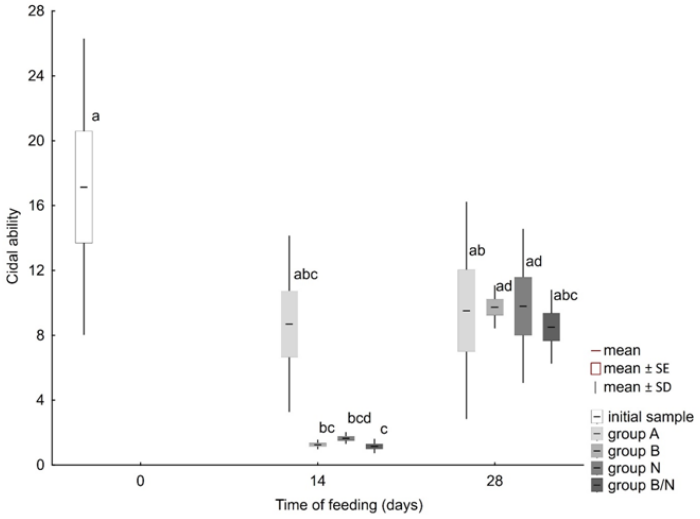



Figure 3. Phagocytic cidal ability of juvenile sea trout fed formulated feeds (groups A and B), the mixed diet (feed B and prey fish; group B/N), prey fish (group N) confirmed after 0, 14, and 28 days of the trial expressed as the ratio (product) of myeloperoxidase activity and the number of actively absorbing phagocytes. Details in Material and methods. Values with different letter indexes differed significantly at P≤0.05

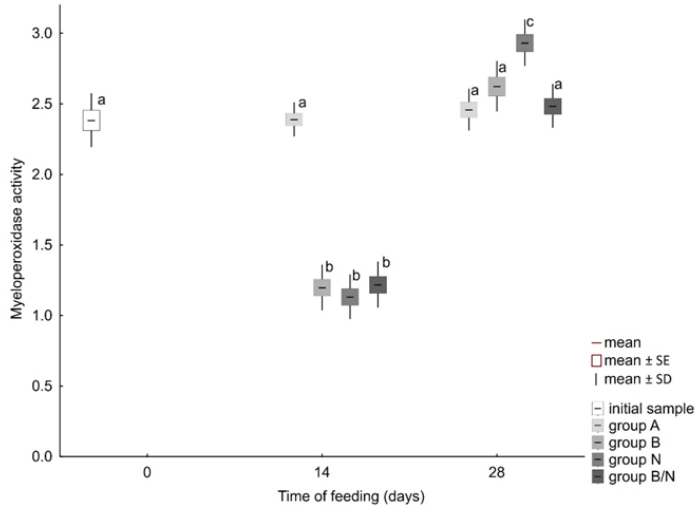



Figure 4. Myeloperoxidase activity in phagocytic cells of juvenile sea trout fed formulated feeds (groups A and B), the mixed diet (feed B and prey fish; group B/N), prey fish (group N) confirmed after 0, 14, and 28 days of the trial. Details in Material and methods. Values with different letter indexes differed significantly at P≤0.01

**Stress indicators**

The type of feed significantly affected concentrations of glucose (F = 42.4; P<0.001) and lactate (F = 10.3; P<0.001). After two weeks, significantly higher GLU values were noted in groups A, B, and B/N (Table 7). After four weeks of the test, GLU levels stabilized and did not differ significantly from those in initial fish sample. Cortisol levels were significantly influenced by both diet (F = 5.4; P<0.01) and the duration of its provision (F = 17.9; P<0.001; interactions between D and T were insignificant). A significant increase in cortisol concentration in the first two weeks of the experiment was noted in group B. After four weeks, decreased cortisol levels comparable to those noted in the initial fish sample were confirmed in groups N and B/N. The analysis of the content of $Na^+$ ions indicated that the diets had a significant influence on them (F = 15.0; P<0.001) as did the duration of their provision (F = 3.9; P<0.05) and the interactions between these two factors (D×T; F = 2.5; P<0.05). The concentration of $K^+$ ions was significantly determined by diet (F = 13.5; P<0.001), test time (F = 15.8; P<0.001), and interactions between D×T (F = 7.8; P<0.001). Increased $K^+$ ion values were noted after two weeks of the test in fish from group N and after four weeks in groups N and B/N. However, no differences among groups were noted in the concentrations of $Cl^-$ ions (P>0.05; Table 7).

Table 6. Results of ANOVA and two-factor ANOVA analyses of the biochemical parameters of juvenile sea trout fed different feeds

| Parameter | Time of feeding (days) | Feeding groups | | | | Factor effect significance (F statistic and P value) | | | Model fit goodness | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | group A | group B | group N | group B/N | feed (D) | feeding time (T) | D × T | $R^2$ | F statistic and P-value |
| TG | 0 | 182 b±21 | | | | F = 13.3 P<0.001 | F = 1.6 P = 0.18 | F = 6.0 P<0.001 | 0.41 | F = 6.2 P<0.001 |
| | 14 | 226 ab±32 | 288 ab±53 | 430 a±146 | 243 ab±74 | | | | | |
| | 28 | 245 ab±60 | 272 ab±77 | 194 ab±79 | 389 ab±215 | | | | | |
| CHOL | 0 | 312 b±55 | | | | F = 54.6 P<0.001 | F = 28.1 P<0.001 | F = 8.5 P<0.001 | 0.74 | F = 22.3 P<0.001 |
| | 14 | 311 b±21 | 375 b±43 | 584 ac±118 | 456 bc±103 | | | | | |
| | 28 | 383 b±48 | 402 b±64 | 705 a±66 | 658 a±169 | | | | | |
| TP | 0 | 3.14 b±0.17 | | | | F = 57.7 P<0.001 | F = 1.1 P = 0.37 | F = 2.1 P = 0.06 | 0.59 | F = 11.9 P<0.001 |
| | 14 | 4.3 a±0.87 | 3.79 ab±0.24 | 3.93 a±0.31 | 3.89 a±0.25 | | | | | |
| | 28 | 3.99 a±0.36 | 4.01 a±0.36 | 4.21 a±0.38 | 4.50 a±0.51 | | | | | |
| ALB | 0 | 1.84 c±0.10 | | | | F = 59.7 P<0.001 | F = 0.99 P = 0.40 | F = 0.5 P = 0.79 | 0.58 | F = 11.0 P<0.001 |
| | 14 | 2.16 ab±0.08 | 2.12 bc±0.19 | 2.01 bc±0.11 | 2.20 ab±0.15 | | | | | |
| | 28 | 2.31 ab±0.19 | 2.31 a±0.20 | 2.24 ab±0.28 | 2.28 ab±0.15 | | | | | |
| GLB | 0 | 1.31 b±0.12 | | | | F = 23.0 P<0.001 | F = 1.2 P = 0.33 | F = 2.57 P<0.05 | 0.39 | F = 5.9 P<0.001 |
| | 14 | 2.16 a±0.86 | 1.67 ab±0.18 | 1.92 ab±0.33 | 1.69 ab±0.25 | | | | | |
| | 28 | 1.68 ab±0.20 | 1.70 ab±0.20 | 1.97 ab±0.34 | 2.22 a±0.60 | | | | | |
| BIL-T | 0 | 0.56±1.04 | | | | F = 6.0 P<0.01 | F = 0.001 P = 1.0 | F = 0.01 P = 1.0 | 0.01 | F = 1.1 P = 0.38 |
| | 14 | 0.15±0.10 | 0.10±0.03 | 0.11±0.14 | 0.10±0.05 | | | | | |
| | 28 | 0.03±0.01 | 0.05±0.04 | 0.04±0.03 | 0.06±0.04 | | | | | |
| AMON | 0 | 249±87 | | | | F = 1.5 P = 0.22 | F = 0.33 P = 0.81 | F = 0.56 P = 0.76 | 0.05 | F = 0.67 P = 0.76 |
| | 14 | 320±92 | 282±67 | 288±73 | 254±100 | | | | | |
| | 28 | 268±66 | 339±199 | 262±42 | 289±95 | | | | | |
| ALT | 0 | 3.1 ab±3.1 | | | | F = 10.0 P<0.001 | F = 2.6 P = 0.06 | F = 0.87 P = 0.52 | 0.21 | F = 3.02 P<0.05 |
| | 14 | 11.7 ab±9.7 | 11.9 a±3.9 | 7.3 ab±5.9 | 4.7 ab±4.5 | | | | | |
| | 28 | 6.4 ab±5.7 | 5.7 ab±3.7 | 3.9 ab±6.7 | 2.4 b±2.4 | | | | | |
| AST | 0 | 422±49 | | | | F = 0.48 P = 0.62 | F = 1.3 P = 0.28 | F = 0.40 P = 0.87 | 0.05 | F = 0.66 P = 0.77 |
| | 14 | 417±121 | 423±82 | 369±121 | 375±128 | | | | | |
| | 28 | 435±152 | 465±72 | 379±149 | 365±88 | | | | | |
| ALP | 0 | 229 ab±49 | | | | F = 5.9 P<0.01 | F = 2.9 P<0.05 | F = 0.86 P = 0.53 | 0.15 | F = 2.33 P = 0.02 |
| | 14 | 187 b±33 | 243 ab±49 | 240 ab±54 | 204 b±26 | | | | | |
| | 28 | 233 ab±49 | 302 a±86 | 265 ab±43 | 249 ab±35 | | | | | |

Mean values ± SD (n = 7). Parameter values with different letter differ significantly (one way ANOVA; P≤0.05). A P value lower than 0.05 indicated the factor had a significant effect. Group A – formulated feed (Skretting®); group B – formulated feed (BioMar®); group B/N – mixed feed (BioMar® and prey-fish); group N – prey fish. Details in Material and methods. TG – triglycerides (mg/dL); CHOL – cholesterol (mg/dL); TP – total protein (g/dL$^1$); ALB – albumin (g/dL); GLB – globulin (g/dL); BIL-T – total bilirubin (mg/dL); AMON – ammonia (µg/dL); ALT – alanine aminotransferase (U/L); AST – aspartate aminotransferase (U/L); ALP – alkaline phosphatase (U/L).

Table 7. Results of ANOVA and two-factor ANOVA analyses of stress indices of juvenile sea trout fed different feeds

| Parameter | Time of feeding (days) | Feeding groups | | | | Factor effect significance (F statistic and P value) | | | Model fit goodness | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | group A | group B | group N | group B/N | feed (D) | feeding time (T) | D × T | $R^2$ | F statistic and P-value |
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
| CORT | 0 | 0.58 b ±0.57 | | | | F = 5.04 P<0.01 | F = 17.9 P<0.001 | F = 1.7 P = 0.15 | 0.52 | F = 5.6 P<0.001 |
| | 14 | 5.62 ab±3.10 | 8.24 a±2.57 | 4.67 ab±3.50 | 3.01 ab±3.15 | | | | | |
| | 28 | 4.40 ab±0.62 | 3.12 ab±2.54 | 0.53 b±0.30 | 0.03 b±0.03 | | | | | |
| GLU | 0 | 73.9 c±9.8 | | | | F = 42.4 P<0.001 | F = 2.1 P = 0.10 | F = 5.3 P<0.001 | 0.57 | F = 11.2 P<0.001 |
| | 14 | 108.0 ab±20.8 | 121.0 a±16.6 | 84.6 bc±6.6 | 97.1 b±17.2 | | | | | |
| | 28 | 80.0 bc±10.7 | 78.7 bc±8.6 | 87.1 bc±5.5 | 87.0 bc±7.6 | | | | | |
| LACT | 0 | 13.1± 5.6 | | | | F = 10.3 P<0.001 | F = 1.2 P = 0.32 | F = 0.62 P = 0.71 | 0.17 | F = 2.5 P<0.05 |
| | 14 | 27.2±18.5 | 18.9±7.6 | 17.1±6.6 | 21.6±8.7 | | | | | |
| | 28 | 22.5± 4.6 | 20.5±4.6 | 20.1±2.3 | 21.5±4.1 | | | | | |

Table 7 – contd.

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
|---|---|---|---|---|---|---|---|---|---|---|
| $K^+$ | 0 | 2.23 c±0.20 | | | | F = 13.5 P<0.001 | F = 15.8 P<0.001 | F = 7.8 P<0.001 | 0.57 | F = 11.0 P<0.001 |
| | 14 | 2.55 bc±0.34 | 2.71 bc±0.49 | 8.99 a±2.74 | 3.33 bc±0.93 | | | | | |
| | 28 | 2.38 c±0.22 | 2.43 c±0.45 | 6.35 ab±2.85 | 7.50 a±5.20 | | | | | |
| $Na^+$ | 0 | 163 bc±3 | | | | F = 15.0 P<0.001 | F = 3.9 P<0.05 | F = 2.5 P<0.05 | 0.36 | F = 5.2 P<0.001 |
| | 14 | 169 a±4 | 167 abc±4 | 161 c±2 | 164 abc±3 | | | | | |
| | 28 | 168 ab±3 | 169 ab±5 | 166 abc±4 | 169 ab±2 | | | | | |
| $Cl^-$ | 0 | 128±4 | | | | F = 1.0 P = 0.38 | F = 2.0 P = 0.12 | F = 1.0 P = 0.43 | 0.03 | F = 1.3 P = 0.26 |
| | 14 | 127±1 | 128±2 | 130±4 | 125±2 | | | | | |
| | 28 | 127±2 | 127±2 | 127±4 | 125±3 | | | | | |

Mean values ± SD (n = 7). Parameter values with different letter differ significantly (one way ANOVA; P≤0.05). A P value lower than 0.05 indicated the factor had a significant effect. Group A – formulated feed (Skretting®); group B – formulated feed (BioMar®); group B/N – mixed feed (BioMar® and prey-fish); group N – prey fish. Details in Material and methods. CORT – cortisol (ng/mL); GLU – glucose (mg/dL); LACT – lactate (mg/dL); $K^+$ – potassium ions (mmol/L); $Na^+$ – sodium ions (mmol/L); $Cl^-$ – chlorine ions (mmol/L).

## Discussion

Changes in fish rearing conditions in aquaculture facilities, including diets, are stress factors that can affect various physiological processes in fish, and, consequently their vitality and rearing indicators (Sadoul and Vijayan, 2016; Sopinka et al., 2016; Ciji and Akhtar, 2021). This includes changes in feeding with formulated feeds with different components and/or of different chemical composition (e.g., changing the brand of the feed or providing functional feeds), and also feeding live feed in periods preceding stocking (Czerniawski et al., 2015; Ahmed et al., 2020). It should be noted that the change in diets in the present experiment was twofold. The first modification was to replace the formulated feed the sea trout received prior to the test (initial fish sample) with salmonid feed from a different manufacturer (group B). The second modification was more drastic since the A feed was replaced with prey fish (groups N and B/N). Importantly, these changes, including the introduction of live feed, either gradually (group B/N) or fully (group N), were not reflected in the values of growth indicators (FL, SL, BW, SGR, DGR). This proved that the cultured juvenile sea trout (fed only formulated feed) accepted the new feed and was evidence of the adaptability of this species. However, the diets tested influenced the condition factor of the fish. The condition factor (CF) in the groups fed only formulated feed (groups A and B) were significantly higher. The energy value of the natural feed was fivefold lower than that of the formulated feed, which, inter alia, resulted in decreased CF values. Fish fed formulated feed deposit excess energy, inter alia, in the body cavity, which changes body proportions and increases CF values compared to sea trout that are fed natural feed (zooplankton, nekton, fish) (this study, Czerniawski et al., 2010). These differences could also be influenced by increased energy expenditures on locomotor activity associated with changes in fish behavior, i.e., catching live feed (Sopinka et al., 2016). In the current experiment, favorable FCR values were obtained for the formulated feeds (approximately 0.9 in groups A and B) that were comparable to the results obtained in feeding tests conducted on juvenile sea trout in which this type of feed was also used (Hoffmann et al., 2021). The FCR values obtained in the sea trout group that was fed mixed feed (formulated feed + prey fish; group B/N) for the first two weeks indicated that this species is characterized by high plasticity and nutritional opportunism, because it consumed both formulated feed and prey fish. In addition to the FCR values, this was confirmed by observations made during the daily rearing procedures conducted each morning during the experiment.

Introducing only natural feed to diets can cause short-term changes in the values of RBC, HGB, HCT, MCH, and MCHC. Diet modification has been demonstrated to affect processes of blood cell formation and function (Ahmed et al., 2020). Changes in the values of these indicators (decreased values of RBC, HGB, and HCT and increased values of MCH and MCHC) can also be explained by the fish starving during the diet modification period (Ahmed et al., 2020; Witeska et al., 2021). However, the daily observations of feed consumption and the rearing indicator values the sea trout obtained indicated that these changes were associated only with the change in diet, especially when transitioning from formulated feed to prey fish (i.e., diametrically different feed, chemical composition, energy value) and broadly understood physiological adaptations to new feed (Ahmed et al., 2020). Importantly, after four weeks, the values of these indicators in all sea trout groups, including group N, returned to the values observed in the other feed groups. The change in diet did not influence WBC counts in the groups of sea trout examined. Important information about the impact of given procedures on the health status of fish is provided not only by the absolute WBC value but also by the leukogram or leukocyte profile, i.e., the relative share of different types of leukocytes (Sopinka et al., 2016). Conclusions regarding the growth potential and vitality of animals can be based on this parameter (Davis et al., 2008). Fazio (2019) found that body responses to

given environmental factors are manifested more quickly by changes in leukograms than by WBC counts. In the current experiment, the percentage of particular types of blood cells did not differ among fish groups throughout the test. Nevertheless, after conversion to absolute values, statistically significant differences were noted in the numbers of lymphocytes and neutrophils. In the present experiment, increases in total lymphocytes (after two and four weeks of the test) and a significant decrease in total neutrophils (after two weeks of the test) were noted only in the groups fed feed B (groups B and B/N). These differences stemmed from changes in the number of small lymphocytes (increased in relation to initial fish sample) and neutrophils with segmented nuclei (decreased numbers). These changes could be explained by immune system activation in the fish from these groups in response to changes in their diets (Witeska et al., 2021). This reaction most likely consisted of the displacement of mature granulocytes into the tissues of the intestine (the organ that came into direct contact with new antigens), which explained their reduced numbers in the circulating blood. Simultaneously, a new pool of small lymphocytes appeared in the blood, which was evidence that the bodies were ready to mount humoral responses to potential infection (Witeska et al., 2021). According to Rombout et al. (2005), immunity in juvenile fish is largely dependent on innate immunity, which remains the dominant component of the immune system throughout life. This is because of the relatively delayed specific response of immunocompetent cells especially at low water temperatures (Magnadóttir, 1998). The fish innate immune system consists of a cell component (phagocytic cells – mainly neutrophils and monocytes) and a humoral component (e.g., agglutinins, precipitins, cytokines, chemokines, antibacterial peptides, etc.) (Magnadóttir, 2006). The latter recognizes the structural components of bacterial cells, fungi, and other microorganisms such as lipopolysaccharides, glucans, and polysaccharides. By combining with them, it facilitates recognition and destruction in phagocytosis. The innate immune system acts as an alarm system that provides time for the humoral immune system to respond (Rodriguez et al., 2020). The results of the present experiment showed that this alarm reaction was recorded particularly clearly in the case of the fish fed feed B, which could have possibly contained new feed components that were previously unknown to sea trout. Since these changes were not noted in the group of sea trout fed prey fish (group N), this could confirm the nature of the sea trout immune system response that was observed. However, it should be emphasized that information about fish leukograms and the characteristics of their leukocytes is only available for a few species (Fazio, 2019). To the best of the authors' knowledge, this does not include sea trout; hence, it is difficult to draw any further conclusions. However, the changes in phagocytic activity recorded in the sea trout blood also supported the reaction mechanism described above, because the percentage of cells actively absorbing zymosan grains and the activity of myeloperoxidase were both clearly lower after two weeks of the test in all the groups in which the diets were changed. This was confirmed by the results obtained from the leukogram, namely the decreased count in the blood of neutrophils with segmented nuclei. These granulocytes are believed to be mature cells capable of phagocytosis and the destruction of microbes (Clauss et al., 2008). These are cells that can move from the bloodstream to other tissues (Havixbeck and Barreda, 2015). The displacement of so many cells from the bloodstream to the intestines certainly diminished body defenses in other tissues, and to estimate how much, we developed an index (cidal ability [CA]) that aggregates the number of active phagocytic cells and myeloperoxidase activity. CA clearly showed that the ability to destroy microbes in the blood decreased significantly after two weeks of feeding experimental feed, but after four weeks it returned to a level close to the initial value.

The influence of both factors tested (D and T) was significant with regard to cortisol levels, but the lack of interactions between them could, to a certain extent, have indicated individual variability in this parameter (Sopinka et al., 2016). It is worth noting that a significant increase in cortisol (from 0.58 to 8.24 ng/mL) was noted in the first two weeks of the test in group B (formulated feed changed from A to B). After four weeks, the lowest cortisol concentration was in the groups that were fed natural feed (groups B/N and N at 0.03 and 0.53 ng/mL, respectively). Generally, the cortisol levels in the sea trout tested (with the exception of group B, fed formulated feed, after two weeks of the test) were considered low. Cortisol levels in unstressed salmonids (sea trout and rainbow trout) range from 0 to 5 ng/mL, while after the occurrence of an acute stress reaction they increase to a range of 40–200 ng/mL (Pickering and Pottinger, 1989). In turn, Pottinger and Moran (1993) reported that normal blood plasma cortisol levels in unstressed rainbow trout are approximately 7.5 ng/mL, and in Atlantic salmon they are 27–99 ng/mL. It should be noted that reactions to stress even in very closely related fish species can differ significantly (Sopinka et al., 2016), just as differences between wild and cultured specimens of the same species can be significant. This phenomenon has also been confirmed in sea trout (Lepage et al., 2000). Thus, the stress reaction is determined by many factors (e.g., the type and strength of the stressor, age, season, nutritional state, ontogenetic developmental stage, domestication) (Sopinka et al., 2016; Ciji and Akhtar, 2021). The data discussed above indicated that changes in diets, particularly to natural feed, did not have a significant influence on juvenile sea trout cortisol levels (Martínez-Porchas et al., 2009; Sopinka et al., 2016; Ciji and Akhtar, 2021). It must be noted that, among other things, the fatty acids profiles of the diets fed to the sea trout differed. Long-chain PUFAs, especially arachidonic acid (AA), are precursors of eicosanoids (e.g., prostaglandins). Prostaglandin modulates the activity of the hypothalamic-pituitary-interrenal (HPI) axis with regard to cortisol release

(Ciji and Akhtar, 2021). It is possible that the different AA levels in the current diets (the lowest were in feed B) could have contributed to differences in cortisol levels among the groups.

The diets provided significantly influenced the values of secondary physiological indicators of stress (glucose and lactate) in sea trout. Increased glucose levels were noted after the fish were fed formulated feed (groups A, B, B/N) for two weeks. Considering that both glucose and lactate stabilized after four weeks of the experiment, the high individual variability of the values of these parameters, and the fact that the concentration of these metabolites is also determined by metabolic processes that are independent of stress, the results cannot be interpreted unequivocally and can only be associated with this phenomenon (Martínez-Porchas et al., 2009; Sopinka et al., 2016). Relatively small differences among groups in the levels of these metabolites (e.g., the maximum increase in glucose level of 63% (group B relative to initial fish sample) could confirm this hypothesis. Under stress conditions involving the stimulation of the HPI axis and/or the release of catecholamines, levels of glucose and lactate often increase manyfold (e.g., Martínez-Porchas et al., 2009; Demska-Zakęś et al., 2021). In the present experiment significant increases in the concentrations of potassium and sodium ions were also noted in sea trout. This phenomenon could be linked with increased gill diffusion capacity that caused changes in plasma osmolality and ion concentrations. This type of body reaction, however, depends on many external and internal factors, which is why it is often difficult to interpret results (Sopinka et al., 2016). Significant increases in $K^+$ ion concentrations in the fish provided natural feed (groups N and B/N) could be explained by changes in metabolism and locomotor activity associated with behavioral changes and catching prey fish. Confirmation of the increased energy expenditure of fish eating prey fish was the slightly lower condition factor value noted in these fish. Thus, this could be associated with changes in the energy budget and bioenergetics of the fish (Beyers et al., 2002).

The contents of cholesterol, triglycerides, total protein, albumin, globulin, and ALP in plasma are measures of the nutritional status of fish; however, for example, changes in the concentration of specific protein fractions can be indicators of fish health (Folmar, 1993; Sala-Rabanal et al., 2003). According to Deng et al. (2013), blood cholesterol reflects the level of the *de novo* synthesis of cholesterol in the body, and an excess of cholesterol, regardless of its origin, is eliminated from the body through the increased secretion of sterols and bile acids. However, Zhu et al. (2020) found that high levels of dietary cholesterol suppressed the expression of cholesterol synthesis genes but did not influence cholesterol elimination gene expression in young rainbow trout, and feeding diets containing cholesterol resulted in higher blood cholesterol in the rainbow trout in this experiment. In the present experiment, the sea trout fed natural feed had significantly higher levels of blood cholesterol. Unfortunately, we could not compare the content of cholesterol in the experimental and natural feeds as we did not measure it. However, considering that the experimental feeds contained significant shares of plant-based raw ingredients, we can assume that the supply of dietary cholesterol was probably higher in groups N and B/N, and this led to increased cholesterol in the blood. The low sensitivity of LXR receptors to dietary cholesterol, reported by Zhu et al. (2020), seems to be a logical, natural mechanism in young, fast-growing fish that require cholesterol to build cellular membranes intensively. This mechanism could permit freeing up significant amounts of energy needed for cholesterol synthesis when dietary cholesterol is available in excess. On the other hand, two-factor ANOVA revealed that increased blood cholesterol levels were both dependent on diet and time, and, moreover, interactions between these two factors were noted. This indicated that that blood cholesterol levels increased progressively over time depending on feed type, which suggested that there was either accumulation in the blood, or a rather low level of cholesterol deposition in the tissues.

**Conclusions**

The results of this study indicated that juvenile sea trout can be successfully fed prey fish, for example, during the period prior to stocking. Implementing both "soft" (mixed feed – formulated feed + prey fish) and "hard" (feeding prey fish exclusively) changes in diet did not influence the absolute or relative indicators of fish growth. The lowered condition factors of sea trout fed prey fish in comparison with fish fed formulated feeds was linked, inter alia, with the lower energy content of this diet and with the increased locomotor activity associated with catching prey fish. The experiment also revealed that changes in diet, regardless of their character (whether to another formulated feed or to natural feed), influenced blood parameters (RBC, HGB, HCT, MCH, MCHC), leukograms and leukocyte indices, fish nutritional status parameters (e.g., cholesterol), and stress indicators (e.g., cortisol and glucose). These changes were noted mainly after two weeks of the experiment, but after four weeks of feeding the sea trout with the diets tested, they were eliminated. It is noteworthy that lower myeloperoxidase ($A_{MPO}$) and cidal (CA) activities were noted after two weeks in all the fish groups in which the diets were changed. These parameters returned to values noted before the change in diets after four weeks of feeding, which indicated that feeding sea trout prey fish for too short a time (e.g., two weeks) prior to stocking could be insufficient for the fish to fully adapt physiologically, including immunologically. Consequently, the potential of the rearing procedures aimed at training fish to consume natural feed and the opportunity to improve the vitality of restocking material would not be fully utilized. Therefore, it appears that with juvenile sea trout a longer period of feeding natural feed is recommended before introducing them into natural watercourses (e.g., one month).

**Data availability statement**

The data of this study are available from the corresponding author upon reasonable request.

**Conflict of interest**

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

**Ethical approval**

The study was conducted in strict compliance with the guidelines of the National Ethics Commission (Warsaw, Poland). All experimental procedures were performed in compliance with the guidelines of the Local Ethics Commission of the University of Warmia and Mazury in Olsztyn (northern Poland) regarding minimizing animal suffering in accordance with the recommendations of Polish law and European Union Directive 2010/63/EU (Approval No. 53/2020).

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