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Prolonged cortisol elevation alters whole body and tissue metabolism in rainbow trout (*Oncorhynchus mykiss*)

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ARTICLE INFO

ABSTRACT

Chronic elevation of circulating cortisol is known to have deleterious effects on fish, but information about the consequences of prolonged cortisol elevation on the metabolism of fish is scarce. To test the effects of chronic cortisol elevation on the aerobic performance of rainbow trout, we examined how two severities of chronically elevated plasma cortisol levels affected the oxygen uptake during rest and after exhaustive exercise using a high (HC) and a medium cortisol (MC) treatment. High cortisol doses significantly affected standard (SMR) and maximum metabolic rates (MMR) compared to control fish. In comparison, the medium cortisol treatment elevated maximum metabolic rates (MMR) but did not significantly influence SMR compared to a sham group (S) and control group (C). The medium cortisol treatment resulted in a significantly increased metabolic scope due to an elevation of MMR, an effect that was abolished in the HC group due to co-occuring elevations in SMR. The elevated SMR of the HC-treated fish could be explained by increased *in vitro* oxygen uptake rates (MO_2) of specific tissues, indicating that the raised basal metabolism was caused, in part, by an increase in oxygen demand of specific tissues. Haematological results indicated an increased reliance on anaerobic metabolic pathways in cortisol-treated fish under resting conditions.

1. Introduction

The release of cortisol from the interrenal cells in the head kidney represents a vital step in a cascade of events following stress. The main function of this corticosteroid during the stress response is to mobilize energy reserves to cope with a stressor and regain homeostasis. The release of cortisol in response to acute stress is beneficial to the fish through an upregulation of gluconeogenesis and diversion of energy to essential processes involved in stressor mitigation (Mommsen et al., 1999; Schreck and Tort, 2016). Low circulating levels of cortisol in the unstressed state are critical for sustaining basic life functions, while a rise in circulating cortisol levels following an acute stressful situation plays an adaptive role. However, chronic cortisol elevation leads to adverse adaptations (Schreck, 1993). These maladaptive alterations are energetically costly since energy substrates mobilized by cortisol are reallocated from other essential processes, such as growth, immune function, or reproduction (Schreck and Tort, 2016). Chronic stress, and the associated cortisol elevation, have therefore been suggested as key factors in reduced growth rates of fish (Philip and Vijayan, 2015). Most studies have assessed the short-term metabolic actions of this hormone.

Less is known about long-term effects of elevated cortisol levels on energy metabolism in fish, but preceding studies do not suggest a down-regulation of the higher energy expenditure associated to this corticosteroid, even after several weeks of treatment (Jerez-Cepa et al., 2019; Pfalzgraff et al., 2021). Prolonged cortisol elevations may occur in poorly managed aquaculture environments that cause social stress, crowding, or poor water quality, or during smoltification or spawning migration in wild populations (*e.g.* Barton et al., 1985; Mcbride et al., 1986; Ruiz-Jarabo et al., 2019; Sundh et al., 2019; Vargas-Chacoff et al., 2020). The increased energy requirement caused by prolonged cortisol elevation must necessarily be satisfied either by endogenous reserves or from dietary nutrients. Turnover rates of all macronutrients are generally upregulated to fuel the elevated energy demand caused by high circulating cortisol concentrations. Administration of cortisol by implants in fishes has been shown to provide similar responses as elevated cortisol levels followed by stressors (Vijayan et al., 2003; Aedo et al., 2019) As such, hepatic gluconeogenic, muscle proteolytic, and hepatic and peripheral lipolytic enzyme activities are reportedly increased in response to cortisol administration, resulting in elevated levels of plasma glucose, free fatty acids, and amino acids following cortisol

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treatment (reviewed in Mommsen et al., 1999).

A proxy for energy expenditure is the mass-specific oxygen consumption (MO_2). The average minimum oxygen consumption of an ectotherm animal in an inactive and post-absorptive state is defined as SMR (Chabot et al., 2016), whereas routine metabolic rate (RMR) is the average rate of oxygen consumption at low levels of activity (Metcalfe et al., 2016). Elevated routine metabolic rates in cortisol treated fish, reflecting an increased energetic expenditure, have been shown for a variety of species, including eel (*Anguilla japonica*) (Chan and Woo, 1978), coastal cutthroat trout (*Oncorhynchus clarki clarki*) (Morgan and Iwama, 1996), rainbow trout (*Oncorhynchus mykiss*) (De Boeck et al., 2001), common carp (*Cyprinus carpio*) (Liew et al., 2013), and pumpkinseed (*Lepomis gibbosus*) (Lawrence et al., 2019). However, only two studies have reported increased standard metabolic rates (SMR) due to cortisol implants; i.e.in largemouth bass (*Micropterus salmoides*) (O'Connor et al., 2011), and pumpkinseed (*L. gibbosus*) (Lawrence et al., 2019). Data on the effect of cortisol on SMR beyond 96 h post-implantation are not available. Maximum metabolic rate (MMR) represents the upper limit for aerobic energy metabolism (Norin and Malte, 2011). The difference between the SMR and MMR provides a measure of the total amount of aerobic energy available, the aerobic or metabolic scope (MS), which is the amount of energy that can be used for feeding, growth, and activity. Following exhaustive exercise to elicit MMR, excess post-exercise oxygen consumption (EPOC) is a measure to assess metabolic recovery time and the cost of anaerobic metabolism during exercise and is an indicator of organismal performance (Lee et al., 2003; Brownscombe et al., 2017). Both MS and EPOC are thought to be affected by cortisol (Eros and Milligan, 1996; Pagnotta et al., 1994). Literature concerning the effects of cortisol on aerobic performance and MS are unequivocal. No difference was observed in aerobic swimming performance between cortisol treated rainbow trout employing coconut oil-based implants at a dose of 250 μg g^{-1} fish, resulting in plasma cortisol levels around 200 ng ml^{-1} and untreated fish (Gregory and Wood, 1999). Contrary, aerobic swimming performance in rainbow trout that were fed cortisol coated diets for 90 days with plasma cortisol levels of 20 ng ml^{-1} was reduced compared to a control treatment, suggesting a reduction in MS due to elevated cortisol levels (Johansen et al., 2017). While Lankford et al. (2005) did not observe changes in aerobic swimming performance, they reported reduced MS driven by elevated SMR in green sturgeon after a 28-day chronic daily stress exposure. More recently, Lawrence et al. (2019) were the first to assess SMR, MMR, and resulting MS after cortisol treatment in a teleost fish (*L. gibbosus*) and found combined increases in SMR and MMR at 24 and 96 h after cortisol implant injections, resulting in an unchanged absolute MS. Currently, no information is available on the effects of prolonged cortisol elevation on MMR or MS for salmonid fish.

Based on known energy intake levels and fecal losses, it has been estimated that the energetic cost of growth due to prolonged plasma cortisol elevation increased by 48–82% (Pfalzgraff et al., 2021). The present study aimed to investigate the magnitude of metabolic costs associated with chronically elevated cortisol levels. We hypothesized that the elevated cost of living due to the detrimental effects of long-term elevation in cortisol levels may affect the metabolic scope. To test these hypotheses, the respiratory physiology of juvenile rainbow trout was assessed by measuring SMR and MMR and calculating the resultant MS. While metabolic rate measurements of fish provide information on the energetic expenses of the whole animal, they do not allow for a differentiation of energetic costs to specific tissues, and it remains relatively unknown how stress and cortisol elevation affects the distribution of metabolic energy (Rodnick and Planas, 2016). Hence, to determine whether energy cost associated with chronic cortisol elevation was caused by particular organs, we measured oxygen consumption in different tissues. Since cortisol has been reported to modulate recovery dynamics in fish, we also analyzed EPOC and recovery time after exhaustive exercise. Haematological parameters at rest were evaluated to investigate correlations between metabolic rates and the occurrence

of anaerobic metabolism. Cardiosomatic indices were assessed to estimate if cardiac remodeling elicited by cortisol might have affected oxygen-carrying capacity. Finally, to estimate substrate utilization to fuel increased energy requirement in cortisol-treated fish, we analyzed the proximate composition of fish carcasses, calculated liposomatic indices, and measured total nitrogen excretion rates.

2. Material and methods

All procedures in this study followed Danish and EU legislation (Directive 2010/63/EU) under a permit from the Danish Animal Research Authority (2020-15-0201-00670).

2.1. Animal husbandry

Juvenile rainbow trout, *O. mykiss* (Walbaum, 1792) were obtained from a commercial producer in Denmark, transported to the aquaculture facilities at DTU Aqua in Hirtshals. Before the trials, the fish were reared in 250 l tanks at 15 °C in a flow-through system, under a photoperiod regime of 15 h light: 9 h dark and constant inlet water flow at a rate of 60 l h^{-1}. Oxygen levels were kept above 85% saturation at all times. Fish were maintained on a commercial diet (EFICO Enviro 920 Advance, BioMar A/S, Denmark) at a daily ratio corresponding to 1.4% of the estimated biomass.

2.2. Treatment groups

Four experimental treatment regimes were used. These are referred to as untreated control (C); sham treatment (S); medium dose cortisol treatment (30 μg cortisol g^{-1} fish, MC); and high dose cortisol treatment (60 μg cortisol g^{-1} fish, HC). Cortisol was i.p. injected using coconut oil as a slow-release implant, as outlined in Pfalzgraff et al. (2021). Briefly, 6 mg (MC) or 12 mg (HC) cortisol (11β, 17α, 21-Trihydroxypregn-4-ene-3,20-dione; Sigma-Aldrich, Canada) was dissolved in 1 ml of melted coconut oil and injected at a dose of 5 μl g^{-1} fish, while the sham treatment was injected with liquified coconut oil only. The coconut oil solidified after injection, and cortisol was gradually released from the implants over the following days and weeks.

2.3. Experimental design and procedures

Before metabolic rate determinations after 23 days post implantation, fish were maintained in triplicate tanks (3 tanks per treatment group of 189 l) with an initial average weight of 115.1 ± 0.4 g (mean ± S.E.) at a stocking density of 15 fish per tank. In addition, 15 rainbow trout per treatment intended for *in vitro* tissue oxygen uptake rate measurements and haematological analysis with an initial average weight of 115.7 ± 1.5 g (mean ± S.E.), were stocked into 4 extra tanks (1 tank per treatment). All fish were fed a commercial trout diet corresponding to 1.4% of the tank biomass daily. Tanks received flow-through municipal water, at an inlet flow rate of 40 l h^{-1}. The water temperature was maintained at 15 °C, and oxygen saturation levels were kept at >85%. Photoperiod was maintained at 15 h light: 9 h dark throughout the trial. From day 6 until day 11 post treatment, all fecal samples were collected as described in Pfalzgraff et al. (2021) for nitrogen analysis (ISO 5983-2, 2009) to calculate nitrogen mass balances. 16 days after the injections, the water supply to the tanks of these fish was turned off for 24 h. A pump in the tank ensured water current and aeration by a diffusor. Nitrogen excretion was measured as total ammonia nitrogen (TAN) from water samples collected just before feeding, and 3, 6, and 24 h after feeding. Water samples were filtered (0.2 μm, Filtropur Sarstedt, Numbrecht, Germany) and stored at 4 °C until total nitrogen analysis (ISO, 1997; ISO, 1986). Results from total nitrogen excretion were used to calculate nitrogen mass-balances as outlined by Lund et al. (2011). Subsequently, water flow was turned on again and fish were reared under before described conditions. 18 days

post-implantation, 10 fish per tank were removed from each tank and sampled for another study (Pfalzgraff et al., 2021). Subsequently, the remaining 15 fish of each treatment were merged in one tank per treatment and reared together until metabolic rate measurements in individual chambers on 8 randomly selected fish per treatment.

2.4. Metabolic rate and EPOC determinations

23 days post-implantation, mass-specific oxygen consumption rate, (MO_2, mg O_2 kg^{-1} h^{-1}) was measured using computerized intermittent flow-through respirometry as described by Steffensen (1989). Feed was withheld for 48 h before MO_2 measurements. A total of 32 rainbow trout with an average body mass of (C) 164.0 ± 6.1 g ($n = 8$); (S) 165.8 ± 8.7 g (n = 8); (MC) 117.1 ± 5.2 g (n = 8); (HC) 127.5 ± 6.1 g (n = 8) (mean $\pm$ S.E.) were netted from their holding tank, weighed and transferred to 20 l buckets containing 10 l of water, in which fish were subjected to a manual chase protocol. To elicit the maximum metabolic rate of the fish, escape responses were triggered by repeatedly touching the tail of the fish for 3 min, at which point the fish were completely unresponsive to physical stimuli. Individuals were then rapidly transferred to a 5.71 l acrylic respirometry chamber immersed in a 250 l container, as described in Skov et al. (2011). The experimental temperature was kept at 15 °C, and the light cycle was identical to that during rearing (15 L: 9 D). MO_2 measurements were conducted in 6 min loops, consisting of a 90s flush period, a 30s wait period, and a 240 s measurement period, using automated respirometry software (AutoResp, Loligo Systems, Tjele, Denmark). The first oxygen measurement period began within 1 min after the end of the chase protocol of each individual. Maximum metabolic rate (MMR) was considered to be the highest oxygen consumption measurement recorded during a measurement cycle. For the estimation of standard metabolic rate (SMR), which was conducted similar to the procedures outlined by Skov et al. (2011), fish stayed in the respirometers for 24 h, resulting in 240 oxygen consumption measurement periods per fish. The measurements were binned in 10 mg intervals to create a frequency distribution. SMR was calculated as the sum of the product of the highest frequency and its class mean plus the product of the three adjacent frequency classes on either side, divided by the total number of observations. The absolute metabolic scope (MS) was calculated as the difference between MMR and SMR values of individual fish, wheras the factorial metabolic scope was calculated as the quotient from MMR and SMR. Excess post-exercise oxygen consumption (EPOC), was calculated as the summated MO_2 for each animal during the period between being placed in the respirometer and being within 5% of their SMR less the summated SMR values during that period. Recovery time was defined as the duration from placing the fish into the respirometer until the fish was within 5% of its SMR.

After the oxygen consumption measurement procedure, fish were euthanized in benzocaine (0.1 g L-1 ethyl-p-aminobenzoate), fork length was measured to calculate condition factor (CF = body weight (g) / fork length^3 (cm)), and individuals were weighed. Subsequently, viscera were detached, and visceral fat was weighed to calculate the liposomatic index. Liposomatic index was calculated as (visceral fat weight (g) / body weight (g)) × 100%. Hearts from all experimental fish were excised, the bulbi and atria were removed, ventricles were blotted dry of blood and weighed on a precision scale to calculate cardiosomatic index (ventricle weight (g) / body weight (g)) × 100%. Following evisceration, the remaining carcasses of the fish were analyzed for their proximate composition.

2.5. Proximate composition analysis after 2 days fasting and 24 h MO_2 measurement

After metabolic rate measurements, the 8 fish per treatment were pooled into 4 samples consisting of 2 fish each. The pooled carcasses were autoclaved for 1 h (120 °C), homogenized, and analyzed for dry matter and ash (NKML, 1991), crude protein (ISO 5983-2, 2009), and

crude lipid (Bligh and Dyer, 1959).

2.6. Tissue oxygen consumption

At 23 days post-implantation, fish for tissue respiration determinations were quickly netted from their holding tanks and transferred to a benzocaine solution (0.1 g l-1 ethyl-p-aminobenzoate) for 20 s. A 2 ml blood sample was rapidly taken from the caudal vessel using 2.5 ml heparinized syringes with 23 G needles, of which one half was transferred to an Eppendorf tube, and immediately spun at 5000 *g*. The supernatant plasma was transferred to −80 °C until plasma cortisol analysis using commercial enzyme-linked immunosorbent assay (ELISA) kits (Neogen Corporation, Lexington, USA) according to the kit protocol. The plates were read in a micro plate reader (CLARIOstar Plus, BMG Labtech GmbH, Ortenberg, Germany). The other half of the blood sample was used for the analysis of haematological parameters. After blood sampling, fish were weighed and placed on ice for tissue dissection. Ventricle, liver, gill filaments, white muscle, head kidney intestine, and stomach were dissected out on chilled petri dishes placed on ice. Intestine and stomach tissue were freed of any visceral fat. Approximately 150 mg of gill filaments, liver, ventricle, head kidney, intestine, and stomach tissue, and 600 mg of white muscle tissue were rinsed with 1 ml ice-cold ringer solution (containing in mmol 123.02 NaCl, 4.96 KCl, 0.93 $MgSO_4$, 3.58 $NaHCO_3$, 5.56 $C_6H_{12}O_6$, 1.46 $CaCl_2$, 7.01 Na_2HPO_4, and 1.05 NaH_2PO_4) as described by Itazawa and Oikawa (1983), and immediately scissored 80 times within 30 s. Gill filaments and white muscle tissue preparations were transferred into glass respirometer tubes of 7.16 ml volume, while liver, ventricle, head kidney, intestine, and stomach tissue preparations were transferred into tubes of 5.45 ml volume. Since mitochondrial enzyme activities were formerly reported to decrease from the proximal to the distal section of the intestine in rainbow trout (Mommsen et al., 2003), the proximal part of the intestine, just posterior to the pyloric caeca, was dissected for tissue respiration measurements. In the stomach, the highest enzymatic activity has been described for the middle part (Mommsen et al., 2003); hence, the tissue samples were cut from this section. After the tissue preparations were placed in the respirometer tubes, they were filled with the ringer solution thermostated at 15 °C, and sealed. The time from netting the fish until the respirometer tubes containing the tissue preparations and ringer solution were closed did not exceed 40 min. Preliminary investigations to obtain a protocol for the tissue respiration measurements showed that no significant differences in tissue oxygen consumption occurred up to one hour after death (authors' personal observations). The respirometer tubes were immersed in a 10 l bath, which was connected to a 100 l water reservoir supplied by tap water and thermostated at 15 °C. Magnetic stir bars set to 1000 rpm in the respirometer tubes ensured appropriate mixing of the ringer solution during the oxygen consumption measurements. Oxygen content in the respirometer was measured every second using a fiber optic oxygen sensor (OXY-4 mini, Precision Sensing GmbH, Regensburg, Germany) collected by automated respirometry software (AutoResp, Loligo Systems, Tjele, Denmark). After a 10 min stabilization period, oxygen consumption measurements were made in 1-min series for 10 min. The software used the slope of the oxygen content within the tubes during the 1 min measurement period, to calculate the oxygen consumption as µg O_2 g^{-1} h^{-1}. Since the decline of oxygen in the tubes during the 10 min was linear, the oxygen consumption of tissues was calculated as the average of the 10 consecutive MO_2 measurements.

2.7. Haematological parameters

Blood pH was measured by a pH electrode immersed in the blood sample using Hanna Instruments PH/ORP/ISE Meter HI 98185 (HannaNorden AB, Kungsbacka, Sweden). To assess glucose levels, 5 µl of blood was collected with a HemoCue micro cuvette and analyzed by HemoCue Glucose 201 RT device (HemoCue AB, Ängelholm, Sweden).

Blood lactate levels were determined by applying 5 µl of blood on a BM-Lactate strip, which was analyzed by Accutrend Plus device (Roche Diagnostics Deutschland GmbH, Mannheim, Germany). Values that were below the detection limit of the device (0.8 mmol l^{-1}) were reported as 0.7 mmol l^{-1}. Additionally, for haematocrit determination, a small amount of blood was centrifuged in 70 µl capillary tubes in a centrifuge with a microhaematocrit rotor (Sigma 1–16, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) for 5 min, photographed and measured digitally. Red blood cell count (RBC) was determined by diluting 10 µl blood into 1 ml trout ringer (containing in mmol 124 NaCl, 3 KCl, 0.75 CaCl$_2$, 1.30 MgSO$_4$, 1.24 KH$_2$PO$_4$, and 12 NaHCO$_3$) (Skov et al., 2011). A further 100 µl blood sample was transferred to a 0.1 mm deep Bürker counting chamber (Paul Marienfeld GmbH, Lauda-Königshofen, Germany), and a minimum of one hundred erythrocytes were counted at 40× magnification. Haemoglobin concentration (Hb) was measured spectrophotometrically at 540 nm following lysis and dilution of red cells in modified Drabkin's reagent (200 mg K$_3$Fe(CN)$_6$, 50 mg KCN l^{-1}, pH 9.6) (Skov et al., 2011). Mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), and mean corpuscular volume (MCV) were calculated from Hb, haematocrit, and red blood cell count values according to Wells et al. (2005).

2.8. Statistical analyses

Statistical analyses were performed using SigmaPlot (version 14.0, Systat Software Inc., Germany). Effects of treatments were tested using a one-way ANOVA, all pairwise multiple comparisons employing the Holm-Sidak method. For evaluation of significant differences caused by treatment on nitrogen mass balances, the Kruskal-Wallis test was applied followed by Tukey test as all pairwise multiple comparison procedure. Differences between treatments were considered significant when $P \le 0.05$. All data are presented as mean ± S.E.

3. Results

3.1. Nitrogen mass balances

Nitrogen mass balances are presented in Fig. 1. Nitrogen retained in rainbow trout was significantly affected by cortisol treatments ($P < 0.001$), also with differences between the two cortisol dosages, whereas no differences were observed between the control and the sham group.
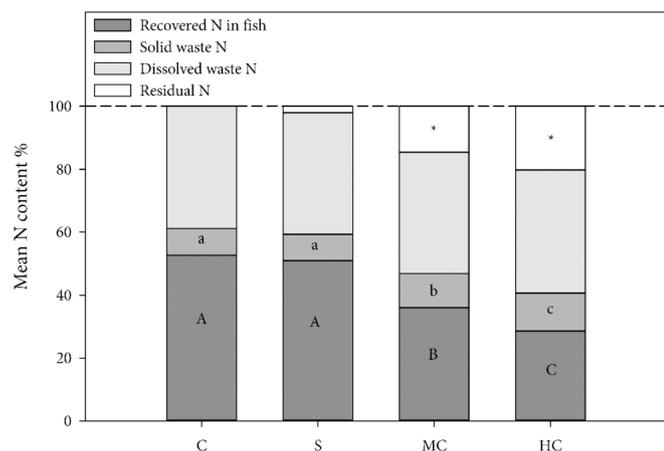
Fig. 1. Mean nitrogen (N) mass-balance from rainbow trout of the different treatment groups ($n = 3$). C: control group; S: Sham group; MC: Medium cortisol dose group; HC: High cortisol dose group. Data are adjusted to 100% of the nitrogen feed intake of the control group. Values not sharing a common superscript letter are significantly different. Asterisks indicate significant differences in residual nitrogen compared to C and S groups.

More nitrogen was recovered in solid waste (faeces) in both cortisol-treated fish groups compared to control and sham groups ($P < 0.001$). There was no statistical difference between treatments in dissolved waste nitrogen. Residual nitrogen (nitrogen not accounted for) was significantly affected by cortisol causing dose-dependent higher residual nitrogen rates in the cortisol treated fish compared to control and sham treatments ($P < 0.001$).

3.2. SMR, MMR, and MS

SMR ($P = 0.003$), MMR ($P = 0.002$), and MS ($P = 0.01$) were significantly altered by cortisol treatments, while none of the observed parameters differed significantly between sham and control fish. Oxygen uptake rates are presented in Fig. 2. SMR was highest for the HC treatment as compared with the sham and control groups. MMR was significantly elevated in the MC treatment compared to C and S treatments, while MMR of the HC group was only significantly different from control fish. C and S groups did not differ significantly among each other. MS was significantly elevated in the MC treated fish compared to control and sham fish, while no differences were observed between MC and HC treatment. When calculating the factorial metabolic scope (fMS), significant differences between treatments disappeared.

3.3. EPOC and recovery time

EPOC and recovery time are shown in Fig. 3. EPOC was not significantly different among treatments, while the recovery time was significantly lower for the HC-treated fish than for all other treatments ($P = 0.009$).

3.4. Carcass composition, condition factor, and organ indices

The proximate composition of the fish carcass samples, organ indices, body weight, and condition factor are presented in Table 1. Dry matter content was significantly higher for HC fish compared to the control group. While there were no significant differences among treatments for ash or protein content, the HC treatment led to a significant decrease of carcass lipids compared to the sham group. MC treatment resulted in significantly reduced lipid content compared to sham and control fish. HC treatment and MC treatment additionally resulted in lower condition factors compared to control and sham fish. The relative mass of the ventricle (cardiosomatic index) was significantly increased for HC treated fish compared to sham and control group, whereas the contribution of visceral fat to the total mass of fish (liposomatic index) was significantly lower for the HC treatment compared to control and sham groups.

3.5. Plasma cortisol levels of individual fish used in in vitro tissue respiration measurements

Cortisol implants in fish designated for tissue respiration measurements (Table 2) resulted in significant elevations of plasma cortisol levels 23 days after hormone implantation. While plasma cortisol levels of control and sham-treated fish showed no differences, MC-treated fish had significantly higher cortisol levels compared to those two groups. HC treatment led to significantly higher plasma cortisol levels compared to all other treatments.

3.6. Tissue oxygen consumption

Oxygen uptake rates of the examined tissues are presented in Table 3. While oxygen consumption of the stomach, intestine, head kidney, and white muscle was not different between groups, cortisol treatment significantly increased the metabolic rate of the liver, ventricle, and gill tissues.
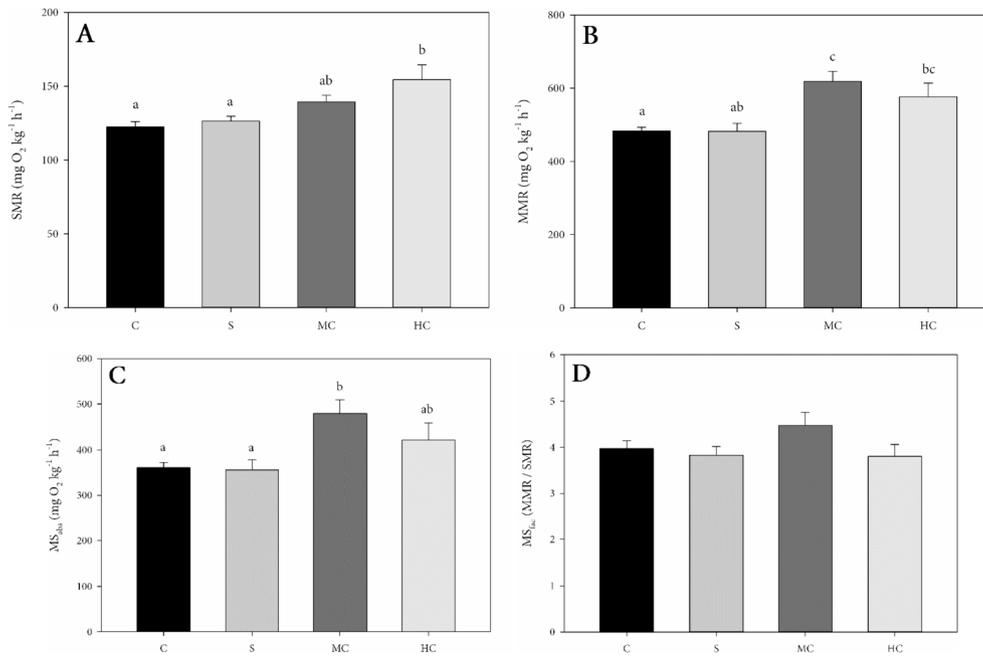
Fig. 2. (A) SMR, (B) MMR, (C) absolute MS (aMS / MS_{abs}), and (D) factorial MS (fMS / MS_{fac}) of experimental fish 23 days post implantation. C: control group; S: Sham group; MC: Medium cortisol dose group; HC: High cortisol dose group. Values not sharing a common superscript letter are significantly different. Values are presented as mean ± S.E., $n = 8$.
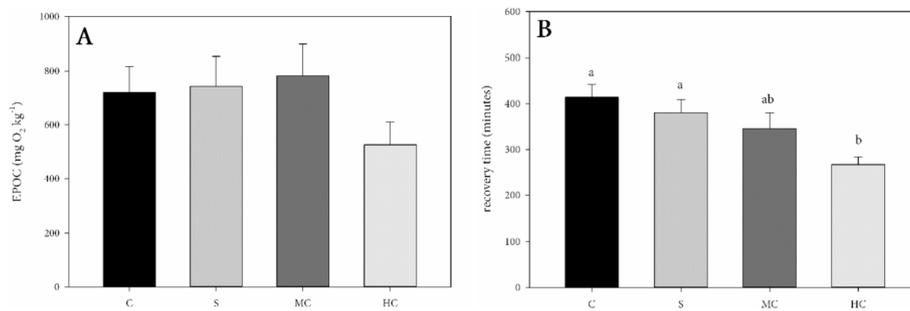
Fig. 3. (A) EPOC and (B) recovery time of experimental fish 23 days post implantation. C: control group; S: Sham group; MC: Medium cortisol dose group; HC: High cortisol dose group. Values not sharing a common superscript letter are significantly different. Values are presented as mean ± S.E., n = 8.

3.7. Haematological variables

Haematological variables at rest are presented in Table 4. No differences were observed in blood glucose levels between treatments. Blood lactate was significantly elevated in the MC and HC fish compared to control and sham-treated fish. Blood pH was significantly lower in both cortisol treated groups compared to non-cortisol treated fish. The same difference between groups was observed in haematocrit, being elevated in cortisol-treated fish. While haemoglobin levels were not affected by treatments, red blood cell count (RBC) was significantly lower in the HC fish compared to the control group. The mean corpuscular volume (MCV), as well as mean corpuscular haemoglobin (MCH), were significantly higher in the HC fish than in sham or control fish. Mean corpuscular haemoglobin concentration (MCHC) was affected by treatments, but the post-hoc test did not reveal any significant differences between the groups.

4. Discussion

4.1. Prolonged cortisol elevation increases SMR and tissue oxygen consumption

Elevation of circulating cortisol levels has previously been shown to increase maintenance costs by mobilizing energy reserves, measured by increased basal metabolic rates of different fish species subjected to cortisol treatment. These metabolic rate increments are comparable to those described for fish facing chronic or reoccurring stress (*e.g.* Lankford et al., 2005; Sloman et al., 2000). Most of the studies reporting higher oxygen consumption rates of cortisol-treated fish carried out metabolic rate measurements within a few hours to days after treatment. Intramuscular cortisol injections of 1 µg g^{-1} fish in the eel (*A. japonica*) led to a 20% increase in oxygen consumption 9–24 h after treatment compared to a control group (Chan and Woo, 1978). Oxygen consumption rates of coastal cutthroat trout (*Oncorhynchus clarkii clarkii*) implanted with 50 µg cortisol g^{-1} fish increased by ~50% 3 days after treatment compared to sham and control treatments (Morgan and Iwama, 1996). Similarly, RMR of common carp (*C. carpio*) was elevated following cortisol implants at a dose of 250 µg g^{-1} fish (Liew et al., 2013). In that study, 7 days after injection plasma cortisol levels of

Table 1

Carcass composition, organ indices, and condition factor 23 days post implantation.

	C	S	MC	HC	P-value
DM	30.62 ± 0.29[a]	31.12 ± 0.35[ab]	31.50 ± 0.15[ab]	31.97 ± 0.07[b]	0.017
Protein (%DM)	56.68 ± 0.49	55.26 ± 0.54	56.58 ± 0.95	55.38 ± 0.22	0.19
Lipid (%DM)	38.71 ± 0.66[ab]	40.37 ± 0.60[a]	35.85 ± 0.77[c]	37.01 ± 0.51[bc]	0.002
Ash (%DM)	8.20 ± 0.16	7.88 ± 0.12	8.66 ± 0.09	8.29 ± 0.30	0.126
Liposomatic index	4.48 ± 0.25[a]	5.07 ± 0.33[a]	2.96 ± 0.35[b]	2.98 ± 0.19[b]	<0.001
Cardiosomatic index	0.105 ± 0.002[a]	0.107 ± 0.001[a]	0.119 ± 0.002[b]	0.134 ± 0.004[b]	<0.001
Body weight (g)	164.0 ± 6.1[a]	165.8 ± 8.7[a]	117.1 ± 5.2[b]	127.5 ± 6.1[b]	<0.001
Condition factor	1.63 ± 0.03[a]	1.67 ± 0.01[a]	1.39 ± 0.03[b]	1.43 ± 0.03[b]	<0.001

DM: dry matter. C: control group; S: Sham group; MC: Medium cortisol dose group; HC: High cortisol dose group. Values not sharing a common superscript letter are significantly different. Values are presented as mean ± S.E., $n = 4$ for compositional analysis; $n = 8$ for organ indices, body weight, and condition factor.

Table 2

Plasma cortisol levels of fish from *in vitro* tissue respiration measurements 23 days post implantation.

	C	S	MC	HC	P-value
Plasma cortisol (ng ml^{-1})	4.1 ± 1.4[a]	3.7 ± 1.3[a]	51.4 ± 7.7[b]	105.8 ± 31.3[c]	<0.001

C: control group; S: Sham group; MC: Medium cortisol dose group; HC: High cortisol dose group. Values not sharing a common superscript letter are significantly different. Values are presented as mean ± S.E., $n = 8$.

Table 3

Mass specific oxygen consumption of several rainbow trout tissues (µg O_2 g^{-1} h^{-1}) 23 days post implantation.

	C	S	MC	HC	P-value
Gill filaments	732.9 ± 14.9[a]	736.2 ± 16.5[a]	845.9 ± 12.5[b]	880.3 ± 21.7[b]	<0.001
Liver	382.3 ± 14.2[a]	385.7 ± 22.3[a]	506.4 ± 19.8[b]	490.9 ± 23.0[b]	<0.001
Ventricle	452.7 ± 22.5[a]	457.0 ± 25.2[a]	515.2 ± 14.8[ab]	566.0 ± 26.2[b]	0.004
Head kidney	432.3 ± 12.1	416.1 ± 28.9	428.0 ± 8.5	480.9 ± 14.0	0.073
White muscle	66.4 ± 3.1	66.6 ± 6.0	72.3 ± 4.1	75.3 ± 6.7	0.554
Intestine	405.2 ± 28.0	352.3 ± 46.0	390.6 ± 22.3	399.7 ± 21.9	0.626
Stomach	417.4 ± 23.0	412.7 ± 16.5	396.4 ± 24.7	414.2 ± 14.0	0.883

C: control group; S: Sham group; MC: Medium cortisol dose group; HC: High cortisol dose group. Values not sharing a common superscript letter are significantly different. Values are presented as mean ± S.E., n = 8.

Table 4

Haematological variables of rainbow trout 23 days post implantation.

	C	S	MC	HC	P-value
Blood glucose (mmol l^{-1})	7.26 ± 0.27	7.20 ± 0.34	6.78 ± 0.29	6.84 ± 0.35	0.602
Blood lactate (mmol l^{-1})	0.81 ± 0.04[a]	0.78 ± 0.03[a]	1.63 ± 0.10[b]	2.08 ± 0.46[b]	0.001
Blood pH	7.58 ± 0.01[a]	7.59 ± 0.02[a]	7.50 ± 0.02[b]	7.50 ± 0.02[b]	0.001
Haematocrit (fraction)	0.37 ± 0.01[a]	0.36 ± 0.02[a]	0.42 ± 0.01[b]	0.40 ± 0.02[b]	0.013
Haemoglobin (g dl^{-1})	13.78 ± 0.50	13.54 ± 0.62	14.35 ± 0.70	13.67 ± 0.47	0.768
RBC (×10^6 mm^{-3})	1.33 ± 0.06[a]	1.27 ± 0.07[ab]	1.20 ± 0.11[ab]	1.04 ± 0.08[b]	0.05
MCV (fl)	277.44 ± 9.37[a]	286.99 ± 14.49[a]	365.16 ± 25.20[b]	396.97 ± 30.07[b]	<0.001
MCH (pg)	104.44 ± 3.86[a]	107.87 ± 5.23[a]	123.77 ± 7.26[ab]	134.33 ± 7.14[b]	0.005
MCHC (g dl^{-1})	37.81 ± 1.35	37.68 ± 0.81	34.17 ± 1.14	34.26 ± 0.90	0.025

RBC: red blood cell count; MCV: mean corpuscular volume; MCH: mean corpuscular haemoglobin; MCHC: Mean corpuscular haemoglobin concentration. C: control group; S: Sham group; MC: Medium cortisol dose group; HC: High cortisol dose group. Values not sharing a common superscript letter are significantly different. Values are presented as mean ± S.E., $n = 8$.

after treatment (O'Connor et al., 2011). Similarly, increased SMR of cortisol treated pumpkinseed (*L. gibbosus*) compared to sham-treated fish was reported, both 24 and 96 h after injections of cortisol in cocoa butter at a dose of 25 µg cortisol g^{-1} fish, which resulted in plasma cortisol levels of approximately 65 and 15 ng ml^{-1} respectively (Lawrence et al., 2019). The findings of these studies prove the importance of the time scale for conducting metabolic rate measurements after treatment with oil-based cortisol implants as the circulating cortisol levels decline over time.

To date, the elevation of SMR due to cortisol treatment has not been reported in fish beyond 4 days post-implantation. This is the first report of elevated SMR in fish measured several weeks after cortisol implantation, and, to our knowledge, also the first study showing elevated SMR in a salmonid fish. Applying two different cortisol dosages, we show a dose-dependent effect of cortisol on the basal oxygen requirement with a 26% elevation (significant) in SMR in the high cortisol dose group, and a 14% elevation (non-significant) in the medium cortisol dose group compared to the control group. Plasma cortisol levels were not determined for the individual fish in the respirometers because measuring metabolic rate itself has shown to increase circulating cortisol levels (Murray et al., 2017). However, the plasma cortisol levels from other individuals in the same treatment groups used for tissue respiration measurements showed that circulating hormone levels were significantly elevated in a dose-dependent manner 23 days post-implantation. The observed increase in SMR after prolonged cortisol treatment demonstrates that the fish were unable to downregulate increased energy expenditure as long as cortisol concentrations were raised, which proves the upregulation of energy-demanding pathways to be a physiological adaptation for as long as circulating cortisol levels are elevated above a certain threshold.

The increased oxygen consumption of certain tissues is conclusively a contributing factor for increased SMR of cortisol-treated fish and proves that elevated metabolic rates are caused by increased energy demand at the tissue level. While several of the studied organ tissues showed elevations in oxygen demand corresponding to the elevated SMR after cortisol treatment, others were unaffected by the hormone. Cortisol has previously been reported to increase tissue oxygen consumption rates of fish, regardless of tissues (Lynshiang and Gupta, 2000). Liver, muscle, kidney, and brain of catfish, *Clarias batrachus*, showed elevated metabolic rates 4 days after daily repeated intramuscular hormone injections of approximately 25 ng cortisol g^{-1} fish. The

treated fish were no longer different from control or sham fish, at which point differences in RMR also ceased. Cortisol implanted rainbow trout also had elevated RMR compared to sham fish in response to elevated plasma cortisol levels (~200 ng ml^{-1}), and changes in MO$_2$ disappeared once cortisol levels returned to normal (De Boeck et al., 2001). In addition to these various studies observing elevated RMRs, we have only found 2 reports of elevated SMR in fish following cortisol treatment, however not more than 4 days after injections. Largemouth bass (*M. salmoides*) injected with cortisol in coconut oil with a dose of 50 µg g^{-1} fish showed an 18% increased SMR compared to control fish 56 h

elevated oxygen uptake rates from *in vivo* studies due to cortisol treatment were supported by results from *in vitro* cortisol administration. Furthermore, tissue oxidative metabolism decreased after cortisol steroidogenesis blocker treatment using metyrapone, an inhibitor of 11β-hydroxylase. It has previously been shown that cortisol affects cardiac function in rainbow trout by increasing ventriclular mass and myocardial contractility (Farrar and Rodnick, 2004; Johansen et al., 2017). Our findings add to the complexity of cortisol effects on the cardiac muscle, showing that cortisol, in addition to causing ventricular hypertrophy also increases mass-specific oxygen uptake rates of the cardiac muscle, suggesting higher energetic requirements of this tissue. We also observed an elevation in oxygen consumption of the gill filaments. The reason for this is unknown, but may be related to a proliferation of mitochondria-rich cells (MRCs), as was reported in rainbow trout after daily cortisol injections of 4 ng g^{-1} fish for 10 days by Laurent and Perry (1990). Likewise, various cortisol treatments caused increased Na$^+$/K$^+$-ATPase and H$^+$-ATPase activity in the gills of different fish species, conceivably leading to elevated oxygen requirement of this tissue (Dang et al., 2000; Kiilerich et al., 2007; Lin and Randall, 1993; Madsen, 1990; Sunny and Oommen, 2001). Increased oxygen consumption rates were also observed in the livers of cortisol-treated fish in our study. This finding is in agreement with the elevated citrate synthase (CS) activity in the liver of freshwater catfish (*C. batrachus*) following cortisol injection (Tripathi and Verma, 2003), and proves an elevated energy demand of the hepatocytes as a consequence of increased hepatic gluconeogenic enzyme activity (reviewed in Mommsen et al., 1999). An upregulation of ureagenesis, which was previously reported after cortisol treatment, might also have increased the metabolic rate of the liver in cortisol-treated rainbow trout (McDonald and Wood, 2004). In the stomach and intestine of Nile tilapia (*Oreochromis niloticus*) the activity of several mitochondrial enzymes including CS was reported to be upregulated 5 days following cortisol injections (Mommsen et al., 2003). While this finding would imply an elevated metabolic rate in these tissues, we did not observe elevated oxygen uptake rates three weeks post-implantation. While a short-term upregulation of the oxidative metabolism after the hormone injections may have occurred, long-term processes might have counteracted any upregulation during prolonged cortisol exposure, as prolonged cortisol treatment leads to decreased growth rates, poor feed conversion efficiency, and atrophy of digestive tissues (Pfalzgraff et al., 2021). Therefore, downregulation of mitochondrial density and CS activity in the digestive tissues of cortisol-treated fish might have compensated for any prior elevations of this enzyme's activity and consequently oxidative metabolism and could be an adaptation to save energy, which is needed to support more essential functions during cortisol elevation. However, the mechanisms of cortisol's effects on intestinal tissue mass and energy metabolism are yet to be discovered. As for the digestive tissues, prolonged cortisol treatment did not alter the metabolic rate of white muscle or the head kidney, possibly due to a lack of glucocorticoid response elements (GREs), as has been shown for the fish kidney (Mommsen et al., 2003).

The protein content of the fish carcasses did not differ significantly between cortisol treated and untreated fish. However, since cortisol-treated fish grew less than untreated fish while consuming the same amount of food, nitrogen retention was lower in these groups. Due to lower digestive performance, there was more nitrogen in solid waste of cortisol-treated fish (Pfalzgraff et al., 2021). The dissolved nitrogen excretion rates however were not different between treatments, which resulted in unrecovered nitrogen in the MC and HC treated fish. Elevated cortisol levels are known to cause changes in nitrogen metabolism including elevated urea excretion rates, inhibited muscle protein synthesis, increased protein catabolic enzyme activities (*e.g.* McDonald and Wood, 2004; DiBattista et al., 2006; Saulnier et al., 2021). Increased proteolytic activity or reliance on protein for gluconeogenesis would require a deamination step and therefore result in elevated nitrogen excretion rates. Nonetheless, such an increase was not observed in our study, nor in a previous study that compared fish composition and

nitrogen excretion rates after prolonged cortisol treatment (De Boeck et al., 2001). Also in agreement with the latter study was the reduced lipid content of cortisol treated fish, which in addition to the reduced liposomatic indices of cortisol treated fish suggests that lipids were the main nutrient utilized to fuel elevated requirements. As lipid oxidation in the cortisol treatments was increased, some of the amino acids might have been deaminated to use the carbon backbone for lipogenesis. This however would also lead to elevated nitrogen excretion. Hence, the question remains, what the fate of the lost nitrogen was. To address this, further research is required to assess nitrogen mass balances and to reveal the fate of lost nitrogen during cortisol elevation.

4.2. Prolonged cortisol elevation dose-dependently modulates MMR, aerobic scope, recovery time, and anaerobic metabolism

The elevated basal oxygen requirement we observed due to cortisol treatment would necessarily lead to reduced energy available for other activities within the scope for activity if the fish were not able to obtain higher MMR. So far, only one study has tested the effect of cortisol using implants on the MMR and MS of fish (Lawrence et al., 2019). While the magnitude of MMR elevation in their study was considerably greater than that of SMR when cortisol levels were around 65 ng ml^{-1}, MS was not significantly different between treated and untreated fish. In our study, moderately elevated cortisol levels resulted in an elevated metabolic scope due to higher MMR values and showed significantly reduced growth rates and digestive capacity (Pfalzgraff et al., 2021). Even 23 days post-implantation, the growth of cortisol treated fish was diminished as the average weight of these fish was not quite different from the initial weight, supporting the hypothesis that as long as circulating cortisol levels are elevated, physiological consequences prohibit the process of growth. Hence, the growth of cortisol-treated rainbow trout was not restricted by oxygen transport capacity. While some authors propose processes such as growth to be related to the MS (*e.g.* Pörtner and Knust, 2007; Auer et al., 2015), there is an ongoing debate about the ecological relevance of the MS to overall fitness and how much of maximal aerobic scope is necessary to maintain optimal fitness (Clark et al., 2013). Our findings contradict the hypothesis that aerobic scope is directly related to whole-animal performance and fitness by allowing more aerobic energy to be utilized for processes such as growth. Similar mismatches between fish growth performance and maximum metabolic scope were reported in studies testing metabolic scope and growth rates at different temperatures. Specific growth rates of killifish (*Fundulus heteroclitus*) and Atlantic halibut (*Hippoglossus hippoglossus*) were reduced at elevated temperatures while the aerobic scope was highest (Healy and Schulte, 2012; Gräns et al., 2014). The metabolic scope is only a capacity, but its utilization by fish is not yet understood (Farrell, 2016). Metabolic power budgeting could be a tool to understand which metabolic processes included in the aerobic scope for activity are upregulated, and thus lead to reduced growth of fish with elevated circulating cortisol levels. Hence, future research assessing SDA measurements or oxygen consumption during routine activity is required to find out, where some of the energetic resources go that are not invested in growth. While the MS of the MC fish was increased, this was not the case for the HC treatment as SMR was elevated under higher cortisol levels, while the opposite was found for MMR. This finding suggests a possible breaking point for the effect of cortisol on MS with circulating cortisol levels increasing MS by elevating MMR greater than SMR until a certain threshold, while at highly elevated cortisol levels MS is compromised since SMR is continuously increasing, while MMR is not. Another way to express the metabolic scope is by putting more emphasis on the SMR as the denominator is the fMS. The fMS was not different between treatments, proving that the choice of reporting absolute or factorial MS might affect the results. Deciding if MS or fMS is the more appropriate way of interpreting the results requires further research to assess whether activities beyond SMR are more energetically costly with elevated cortisol levels.

The elevated MMR of cortisol-treated fish might have been enabled by increased catecholamine responsiveness of the RBC causing chronic erythrocyte swelling as previously proposed (Reid and Perry, 1991). RBC swelling is induced by activation of Na^+/H^+ exchange and the passive influx of water coupled to Na^+ into the RBC (Chiocchia and Motais, 1989). The initial step of this activation is an interaction between a catecholamine and a high-affinity erythrocyte surface β-adrenoreceptor, which in rainbow trout is 20% more abundant following prolonged cortisol treatment (Reid and Perry, 1991). As a consequence of Na^+/H^+ antiporter activation, the pH inside the RBC increases, while the external pH decreases, which was observed in the blood of our cortisol treated fish. Soivio and Nikinmaa (1981), using oxygen dissociation curves, showed that swelling of the erythrocytes increases the oxygen affinity of haemoglobin as a consequence of the increased internal RBC pH so that it can effectively bind oxygen at the gills and deliver it to the tissues. The observed RBC swelling must have caused the increased haematocrit of cortisol-treated fish since RBC numbers were not elevated. Barton et al. (1987) also reported cortisol-induced increases in resting haematocrit levels in rainbow trout with 53% after 2 weeks cortisol treatment and 54% after 6 weeks cortisol treatment compared to 45% and 43% for control treatment respectively. Furthermore, in a study testing the optimal haematocrit hypothesis by altering haematocrit levels in rainbow trout, MMR peak values were found at levels of 42%, as observed in the MC treated fish in our study (Gallaugher et al., 1995). The haematocrit increase due to RBC swelling, ultimately enhancing respiratory performance, could be part of an adaptation as prolonged cortisol elevation leads to higher metabolic demands.

In agreement with Johansen et al. (2017), we found elevated cardiosomatic indices of cortisol treated compared to untreated fish. A higher relative mass of this organ combined with the finding of enhanced myocardial contractility in cortisol-treated rainbow trout ventricles (Farrar and Rodnick, 2004), suggests an elevated stroke volume and hence cardiac output. Greater cardiac output was formerly reported to be linked to higher MMR in rainbow trout (Claireaux et al., 2005), and could therefore explain higher maximum oxygen extraction rates of cortisol-treated fish in our study. While blood glucose levels were not different between treatments, there was a higher abundance of lactate in cortisol treated fish. Lactate is the preferred metabolic fuel of cardiac muscle, and can support maximum cardiac performance following stressful exercise in rainbow trout, which may additionally support the increased MMR of cortisol treated fish (Farrell et al., 1988; Lanctin et al., 1980). Since cortisol led to increased oxidative metabolism of rainbow trout ventricles in our study, the elevated circulating lactate in those fish could be used to fuel myocardial contractility. Other highly aerobic fish tissues such as red muscle, kidney, and brain can also utilize lactate as a metabolic fuel (Lanctin et al., 1980; Teulier et al., 2013; Omlin et al., 2014). In accordance with our finding of raised blood lactate levels due to prolonged cortisol treatment, De Boeck et al. (2001) reported elevated plasma lactate levels of rainbow trout 10 days after coconut oil implant injections containing cortisol doses of 250 ng g^{-1} fish, with corresponding plasma cortisol levels of around 200 ng ml^{-1}. Further evidence supporting increased lactate production due to cortisol treatment was an increased LDH activity in skeletal muscle of gilthead sea bream (*Sparus aurata*) together with increased plasma lactate levels after cortisol injections (Aedo et al., 2019). Lactate efflux from the muscle into the blood is elicited by exhaustive exercise, which was our chosen method to obtain MMR, and lactate clearance contributes to the slow component of the EPOC (Wood, 1991). While the EPOC showed a trend to be lower in HC fish compared to the other groups, the differences due to high variations were not significant. However, the recovery time after exhaustive exercise was significantly shorter in the HC fish compared to the other treatments. Faster oxidation and hence turn-over rates of lactate by high metabolically active tissues in the HC fish might have decreased the recovery time. The main reason for a faster recovery of HC fish conceivably was a lack of post-stress plasma cortisol elevation, as previously reported in rainbow trout with prolonged high circulating cortisol levels after acute handling stress (Barton et al., 1987). Supporting evidence that prevention of the post-exercise rise in plasma cortisol is accompanied by a decreased recovery time in rainbow trout was reported in previous studies using cortisol blockers (Eros and Milligan, 1996; Pagnotta et al., 1994).

To conclude, the present study demonstrates that prolonged cortisol elevation exerts numerous effects on metabolism, and lends evidence to the complexity of physiological responses of fish to glucocorticoids. Prolonged elevated cortisol levels do not *per se* impair aerobic performance in rainbow trout; rather moderately elevated cortisol levels increase the aerobic scope, while this effect was diminished at very high circulating cortisol concentrations due to elevated maintenance oxygen requirements. The increased basal metabolic expenditure was associated with elevated oxygen uptake observed in several metabolically active tissues, while MS was conserved by increasing MMR, possibly *via* increases in haematocrit and ventricular mass. Increased reliance on anaerobic metabolism led to elevated lactate levels, which could be utilized to fuel ventricular tissue. Considering fuel utilization for the greater energetic cost carried by prolonged elevated cortisol levels, the results of this study support lipids as the main oxidized energetic resource in rainbow trout, while the fate of lost ingested nitrogen remains to be discovered.

Declaration of Competing 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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