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Cortisol affects feed utilization, digestion and performance in juvenile rainbow trout

(Oncorhynchus mykiss)

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Abstract

To quantify bioenergetic consequences of chronically or acutely elevated plasma cortisol levels, growth rates, feed conversion ratios and digestive performance was investigated in juvenile rainbow trout (Oncorhynchus mykiss) reared at 15°C. Plasma cortisol levels were elevated using coconut oil based implants. A sham group received coconut oil without cortisol, (S), while a low (LC) and high (HC) cortisol group received implants containing 30 or 60 μg cortisol g⁻¹ fish. An additional group of fish was reared under moderate thermal stress (22°C, TS), while acute increases in plasma cortisol were achieved by subjecting fish to a daily stressor by chasing (DS). An undisturbed treatment group, reared at 15°C served as control (C). Although feed intake was not affected by treatments, cortisol treated fish showed reduced growth rate and feed conversion efficiency compared to all other treatments. This was caused, in part, by impaired digestibility of protein, lipids and nitrogen free extract in the HC treatment, leading to a decrease in available metabolizable energy. The observed decrease in lipid digestibility did not appear to be caused by a reduced digestive lipase activity in the pyloric caeca and anterior intestine as this was not significantly different between treatments 90 minutes after feeding. Chronically elevated cortisol levels led to atrophy of the digestive system revealed by a loss in tissue mass of the intestine and pyloric caeca in the high cortisol treatment, as well as an overall decrease in viscerosomatic index. In addition, the energetic cost of growth was higher in both cortisol treated groups compared to all other treatments. The chronic temperature elevation resulted in improved lipid digestion compared to the control treatment, while growth performance was impaired, however apparently not correlated with elevated plasma cortisol levels. A moderate random daily stress event did not result in any changes of the studied parameters. The results obtained suggest both
an atrophic effect of chronically elevated plasma cortisol levels on digestive tissues resulting in impaired nutrient assimilation, less available metabolizable energy, and an increased routine energy expenditure, leading to a higher cost of growth.

Keywords
Chronic stress, digestibility, growth performance, rainbow trout, metabolizable energy, atrophy

1. Introduction

When the dynamic equilibrium of fish is disturbed, the causative factor is commonly referred to as the ‘stressor’. Fish facing a stressor rely on a series of complex regulated physiological and behavioural responses in order to overcome the threat and re-establish homeostasis, referred to as the stress response (Chrousos and Gold, 1992). The secretion of catecholamines and corticosteroids is the primary neuroendocrine response to stress. The release of noradrenaline and adrenaline from chromaffin cells, located in the head kidney in fish due to activation of the brain-sympathetic-chromaffin cell (BSC) axis is a rapid reaction of the neuroendocrine system. Following activation of the hypothalamus-pituitary-interrenal (HPI) axis, the long-term response is the release of corticotropin-releasing factor (CRF) from the hypothalamus. CRF activates the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary, resulting in the production and secretion of glucocorticoids, mainly cortisol, from the head kidney interrenal cells into the bloodstream in fish (Ellis et al., 2012). Since cortisol is the main biomarker of physiological stress in fish, plasma cortisol levels are commonly used as indicator of stress. The release of catecholamines and glucocorticoids leads to secondary responses, such as cardiovascular and respiratory alterations and mobilization of energy reserves. While the primary and secondary responses aid the fish in overcoming a stressor, intense, repeated or chronic stressors may lead to tertiary responses, being deleterious for the organism.

Cortisol serves as a promoter of energy mobilisation, in particular the utilisation of energy reserves through gluconeogenesis, leading to increased blood glucose levels in a variety of teleost species, which prepares the fish to cope with a stressful situation by providing the demand
of energy needed (Mommsen et al., 1999). One vital metabolic reaction to cortisol stimulation is the elevation in liver metabolic capacity, including enhanced amino acid catabolism (Aluru and Vijayan, 2007). Additionally, changes in the lipid metabolism have been reported in fish with elevated cortisol levels. Free fatty acids are more abundant in the plasma of stressed fish (Mommsen et al., 1999), and cortisol has been demonstrated to increase lipolytic enzyme activity in coho salmon (Oncorhynchus kisutch) liver, muscle and adipose tissue, therefore increasing lipid depletion in these organs supporting the role of this corticosteroid to mobilize energy reserves (Sheridan, 1986).

While the release of cortisol following stress of low intensity or short duration might be beneficial for basic life function, the effects at higher and more persistent elevations in concentration become deleterious (Schreck, 1993). Therefore, in fish facing chronically elevated cortisol levels the stress response loses its beneficial character and becomes detrimental, resulting in reduced growth, reproductive dysfunctions, and decreased pathogen resistance (Wendelaar Bonga, 1997). In aquaculture settings, fish may be exposed to a variety of stressors such as repeated handling, poor water quality, inadequate temperature, social subordination and crowding, and those stressors have shown to chronically increase circulating levels of cortisol (e.g. Culbert and Gilmour, 2016; Pottinger and Carrick, 1999; Sundh et al., 2019, 2009). Accordingly, several studies using exogenous cortisol administration in fish have resulted in suppressed weight gain. This reduction of growth has been attributed to the cortisol-induced shift in energy allocation towards restoring homeostasis, thereby reducing the energy availability for growth. In addition, cortisol has been shown to impair feed intake caused by reduced appetite (Madison et al., 2015), and proposed to alter muscle growth regulation by downregulating muscle formation promoters (Sadoul and Vijayan, 2016).

The effect of stress and cortisol on fish metabolism extends beyond compromised growth through energy redistribution, as the consequences of stress and accordingly elevated cortisol levels in the fish alter the intestinal structure and subsequently inhibit digestive functions by reducing nutrient utilization (Sadoul and Vijayan, 2016). Cortisol has shown to mediate several intestinal functions, affecting intestinal fluid transport, epithelial turnover and structure (Sadoul and Vijayan, 2016; Takahashi et al., 2006; Veillette et al., 1995). In the salmonid species Arctic charr (Salvelinus alpinus), the apparent digestibility coefficients of lipid and dry matter of
subordinate fish were reported to be lower compared to dominant fish, possibly due to chronic social stress (Olsen and Ringø, 1999). As environmental stressors can modify the intestinal lining and permeability, reduced digestive capacity could be caused by alterations in the structure of the gastrointestinal tract, as reported in Atlantic salmon (Salmo salar) and rainbow trout (Oncorhynchus mykiss) subjected to 15 minutes of acute stress (Olsen et al., 2005, 2002). Rainbow trout fed with cortisol containing diets showed a higher production of fecal matter compared to control fish, indicating an overall decrease of the digestive efficiency of the gastrointestinal system, possibly caused by alterations of stomach tissue (Barton et al., 1987). Altogether, this supports a possible action of cortisol on the nutrient absorption by modifying structure and function of the digestive system, causing a reduction in the ability to digest food and assimilate nutrients.

Previously, the growth-suppressing effect of chronic cortisol elevation was linked to its action on nutrient absorption by affecting the mucosal layer of the gastrointestinal system in juvenile rainbow trout (Barton et al., 1987). The exogenous administration of cortisol however causing alternations of digestive tissue structures was conducted via hormone-coated pellets. Therefore, this observation might have been a direct effect of cortisol on the mucosal surface layer of the digestive system while entering the gastrointestinal tract. In the current study, cortisol did not enter the digestive system directly by feed uptake, but was i.p. implanted and thus systemically released to the organs over time.

Previous studies have reported increased energy demands, alterations in the structure of digestive tissues, and a potential increase in fecal matter, resulting from stress treatments or artificially elevated circulating cortisol levels in fish. However, to our knowledge, there are no previous studies that have quantified the effects of chronically elevated plasma cortisol levels on the nutrient digestibility and corresponding available metabolizable energy in fish, or calculating the increase in energetic cost of growth in fish facing elevated cortisol levels.

The aim of this study was thus to assess the effect of chronically elevated plasma cortisol levels on nutrient digestibility and hence to quantify available metabolizable energy and energetic cost of growth and compare it with the effect of a moderate daily repeated stress event and a chronic water temperature elevation well above the thermal optimum for rainbow trout. The thermal stress treatment was chosen to reflect peak temperatures occurring during summer months in
trout farms with high water retention times (Skov et al., 2011). To evaluate whether cortisol or different stressors affect the digestibility of lipids by actions on digestive enzymes, we assessed the lipase activity in the anterior intestine and pyloric caeca. In addition, we quantified the relative mass of intestines and pyloric caeca to test if a decreased ability to digest nutrients might result from an atrophy of digestive tissues. We analysed hepatosomatic index, viscerosomatic index and lipase activity in adipose tissue to evaluate if cortisol increased the energy mobilization from storage tissues.

2. Material and methods

All animal procedures used in this study were conducted in accordance with Danish and EU legislation (Directive 2010/63/EU) under permission from the Danish Animal Research Authority (permit number: 2020-15-0201-00670).

2.1. Animal husbandry and treatment groups

The experimental fish for this study, juvenile rainbow trout (*Oncorhynchus mykiss*), were obtained from a commercial producer in Denmark and transported to the aquaculture facilities at DTU Aqua in Hirtshals, where they were kept on a commercial diet for a period of 2 months. Nine days before the start of the experiment, 270 fish were weighed and randomly distributed in triplicates among 18 identical, 189 L, cylindrical–conical, thermoplastic tanks (n = 15) in a modified Guelph setup as formerly described (Dalsgaard and Pedersen, 2011). Six experimental treatment regimes were performed in triplicate groups and are referred to as: A control treatment (C) reared at 15°C; a group reared at 22°C as thermal stress treatment (TS); a sham treatment (S) that was intraperitoneally (i.p.) injected solely with coconut oil; a low (30 μg g⁻¹ fish) cortisol dose treatment (LC); a high (60 μg g⁻¹ fish) cortisol dose treatment (HC); and a group that was subjected to a daily stress event (DS).

2.2. Experimental design

A flow-through design was used with a water flow rate of 40 L h⁻¹ tank⁻¹. Oxygen saturation levels were kept between 85 and 100 %, and a 15 h light: 9 h dark diurnal photoperiod was maintained throughout the trial. The water temperature was kept at 15°C except for the thermal
stress (TS) group, where electrical titanium heaters (TH-500, Aqua Medic, Bissendorf, Germany) were used to elevate the temperature to 22°C. Based on an average estimated FCR, fish were fed identical commercial trout diets (Efico Enviro 920 Advance, BioMar A/S, Denmark) at a daily ration corresponding to 1.4 % of the tank biomass, divided into two portions. The first portion contained 60 % of the daily ration and was fed at 11:00, the second portion containing 40 % was given at 14:00 h. Automated feeders were used, delivering the feed ration over 20 minutes. Fish of the DS treatment were chased for one minute with a stick at different times of the day in a random order at either 9:00, 12:00 or 15:00 h to prevent adaptation to the stressor. The time points were chosen to have the stress event two hours before feeding, or one hour after feeding respectively. After an acclimation period of the experimental conditions of six days, fish of the S, LC and HC treatments were i.p. injected with coconut oil solely or containing the respective cortisol dosages. Before the intraperitoneal injections, fish were anaesthetized in a benzocaine solution (0.04 g L⁻¹ ethyl-p-aminobenzoate) until loss of equilibrium. During the time of injections, the coconut oil / cortisol solutions were stirred by a magnetic stirrer at 28°C to ensure liquification of the oil and an uniform distribution of the cortisol within the oil. For i.p. injections, 5 μl coconut oil g⁻¹ fish was injected using a 1 ml syringe with a 23 G needle. Unconscious fish were held ventral side up to ensure that organs were not penetrated by the needle. The needle was inserted between the pectoral fins and just after piercing through the peritoneum moved caudally approximately 3 cm. At that point, the oil was slowly ejected from the syringe and the needle was carefully removed. To make sure that the coconut oil solidified after injection, fish were briefly put in cold water (5°C) while still being anesthetized and then returned to their respective tanks to recover. While the sham treatment solely received coconut oil injections, the two cortisol treatments were injected with coconut oil containing dissolved cortisol (11β, 17α, 21-Trihydroxy pregn-4-ene-3,20-dione; Sigma-Aldrich, Canada) in a dose of 6 or 12 mg ml⁻¹ respectively, resulting in 30 µg cortisol g⁻¹ fish (LC) or 60 µg cortisol g⁻¹ fish (HC). These doses were selected based on preliminary observations testing several cortisol concentrations to obtain doses physiologically comparable to chronically stressed fish. After injections, fish were left in the tanks to recover for one day before fish of all groups were weighed at day 0 of the experiment.

2.3. Experimental procedures
The digestibility study was performed during a 9 day period after acclimation. The initial mean weight of the fish at day 0 of the trial was 115.1 ± 0.4 g (mean ± S.E.). All uneaten pellets were collected 5 minutes after the end of the feeding and again manually administered to the respective tanks to make sure all pellets were consumed. Feces were collected in sedimentation columns on a daily basis prior to the first feeding. In order to prevent biological degradation between samplings, each sedimentation column was immersed in ice water. Fecal samples from every three consecutive days were pooled and stored at -20°C until chemical analysis, resulting in three periods of fecal samples. Of these, the second and third samples were analyzed for lipid, protein, dry matter, ash and total phosphorus for calculation of ADCs, while the sample of the first period served as back-up. At day 10 of the digestibility study fish of all tanks were bulk weighed to calculate growth parameters and feed conversion ratios. Fish were then returned to their tanks and fed 1.4 % of the tank biomass for five more days before the final sampling. On day 16 of the experiment, 90 minutes after last feeding, eight fish per tank were quickly netted and immediately transferred into a bucket containing an overdose of benzocaine (0.1 g L⁻¹ ethyl-p-aminobenzoate) sufficiently high to kill the fish within seconds to prevent the induction of a cortisol response. From all fish, blood samples were taken from the caudal vein using 1 ml heparinized syringes with 23 G needles. The whole procedure from netting the fish until all blood samples were taken did not exceed five minutes. The blood samples were immediately spun at 5,000 g and the plasma was transferred to a tube that was immediately transferred to -80°C until analysis. All i.p. injected fish were dissected to verify the presence of a coconut oil implant. 3 % of the i.p. injected fish did not have an implant at the time of dissection and these were excluded from the statistical analyses. Subsequently, fish were iced and three fish per tank were dissected for the digestive tract including intestine, mesenteric fat and pyloric caeca for latter lipase activity determination. Intestines were rinsed and flushed with saline solution (0.9 % NaCl) and samples were stored at -80°C until analysis. The five remaining fish per tank were used to calculate viscerosomatic index (VSI) including visceral fat, hepatosomatic index (HSI) and intestinal-somatic index (ISI) by weighing the whole body, and viscera, liver and intestine with pyloric caeca, respectively. To obtain the ISI, the anterior intestine was cut just posterior to the stomach with the pyloric caeca. All mesenteric fat was detached from the intestine and pyloric caeca and subsequently the intestine and pyloric caeca were rinsed and flushed with
saline solution (0.9 % NaCl) until all digesta were removed. Following this procedure, the tissues were blotted dry and then weighed.

2.4. Chemical analyses

Fecal samples from the second and third period of the digestibility study were thawed, homogenized using an Ultra Turrax, and analyzed for dry matter and ash (NKML, 1991), crude protein (ISO, 2009); crude protein = N * 6.25), crude lipid (Bligh and Dyer, 1959) and total phosphorus (ISO, 1998). Nitrogen-free extract (NFE) was calculated as dry matter (DM) less the quantity of ash, crude protein, and lipid.

2.5. Plasma cortisol analyses

Plasma cortisol levels were determined 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).

2.6. Lipase activity

Lipase activity was determined in pyloric caeca, anterior intestine and adipose tissue homogenized in 10 volumes (weight/volume) of ice cold distilled water according to a slightly modified spectrophotometric method by Nolasco-Soria et al. (2018), using 4-nitrophenyl myristate solubilized in DMSO (Sigma) as substrate. After homogenization using an Ultra-Turrax, samples were spun at 13,000 g at 4°C for 10 minutes. The liquid extract under the top layer was transferred into a new Eppendorf tube and this procedure was repeated two more times to obtain a clear extract, which was stored at -80°C until enzyme analysis. The reaction mixture for each sample in the flat bottom 96-well microplate contained 50 μl of sodium cholate (40 mg ml⁻¹) as bile salt emulsifier, 130 μl 0.2 M Tris-HCl buffer (pH 8.0) and 10 μl of the respective enzyme extract. To start the reaction, 10 μl of 4-nitrophenyl myristate was added to the mixture, after which the absorbance was read at 405 nm every minute for 10 minutes. The enzyme activity was calculated using a standard curve with the same reaction mixture as for the samples; however, the enzyme extract was replaced by distilled water and the substrate by 4-nitrophenol dissolved in DMSO at varying concentrations. The amount of enzyme that catalyzed the
hydrolysis of 1 μmol of substrate per minute at room temperature was defined as one unit of enzyme activity, expressed per gram of tissue.

2.7. Calculations and statistical analyses

The apparent digestibility coefficients (ADCs, %) of dietary nutrients and minerals, were calculated as:

\[ \text{ADC}_i = 100 \times \left( \frac{C_i - F_i}{C_i} \right) \]

where \( i \) corresponds to dietary protein, lipid, NFE, ash, phosphorus or dry matter, \( C \) is the consumed amount of \( i \), and \( F \) is the fecal loss of \( i \).

Since there were no significant differences within the treatments in ADCs between the second and third sampling period, the statistical analyses were performed on the averages of both periods combined for each tank. Available metabolizable energy (ME, feed intake corrected for fecal loss) was calculated using the content of protein, lipid and NFE of the diet, multiplied by their respective energy densities (protein 23.7, lipid 39.6, and NFE 17.2 kJ g\(^{-1}\)) (Brett and Groves, 1979).

The apparent energetic cost of growth was calculated as ME divided by biomass increase, to obtain an energetic equivalent per gram body mass increase.

The specific growth rate (SGR, \% d\(^{-1}\)) was calculated based on the overall biomass gain in the tanks: 

\[ \text{SGR} = 100 \times \left( \frac{\ln W_t - \ln W_0}{\Delta t} \right) \]

where \( W_t \) refers to the biomass at day \( t \), \( W_0 \) refers to the biomass at day \( t_0 \), and \( \Delta t \) is the number of feeding days (Hopkins, 1992).

The feed conversion ratio (FCR, g g\(^{-1}\)) was calculated based on the biomass weight gain and the feed intake: 

\[ \text{FCR} = \frac{\text{feed intake (g)}}{\text{weight gain (g)}} \]

Hepatosomatic index (HSI) was calculated as HSI = (liver weight / body weight) * 100 %; Viscerosomatic index (VSI) was derived from VSI = (viscera weight / body weight) * 100 %; Intestine somatic index (ISI) was calculated as ISI = (intestine with pyloric caeca weight / body weight) * 100 %.

Statistical analyses were performed using SigmaPlot (version 14.0, Systat Software Inc., Germany). All data were analyzed using a one-way ANOVA, all pairwise multiple comparison by means of the Holm-Sidak method. Differences between treatments were considered significant when \( P \leq 0.05 \). All data is expressed as mean ± S.E.
3. Results

3.1. Growth performance and feed conversion ratio

The weight gain for the six treatments during the 9 days of the digestibility study are presented in figure 1, while SGR and FCR are shown in figure 2. Feed intake was identical for all treatments (13.5 % of initial body weight), while weight gain was significantly affected by the treatments (P < 0.001). WG was not different between the control, sham and DS groups, whereas the TS group gained significantly less weight during the nine feeding days. The LC group had a significantly lower weight gain than the aforementioned treatments, and the fish of the HC treatment gained significantly less weight than fish of all other groups. The same differences between the groups were observed for SGR (P < 0.001) ranging from 1.11 ± 0.05 in the HC group to 2.01 ± 0.03 in the control group. The feed conversion ratio, however with highest values in the HC treatment (1.29 ± 0.06) and lowest (0.68 ± 0.01) in the C group, showed the same differences between treatments (P < 0.001).

Figure 1. Weight gain for the six treatments during the 9 days of the digestibility study. Values not sharing a common superscript letter are significantly different. All values are presented as means ± S.E., n = 3.

Figure 2. SGR and FCR of the six different treatments over the 9 day digestibility trial. Values not sharing a common superscript letter are significantly different. All values are presented as means ± S.E., n = 3.

3.2. Digestibility, metabolizable energy and cost of growth

Apparent digestibility coefficients (ADCs), metabolizable energy and cost of growth are presented in table 1. Lipid digestibility was significantly affected by treatments (P < 0.001) and was highest for the TS treatment, while the LC and HC groups with increasing cortisol levels had the lowest lipid digestibility. Protein digestibility was also significantly different between treatments (P < 0.001) and compared to other groups lower for LC and HC treatments, however with no differences between the two latter mentioned groups. Just as for lipid, protein
digestibility was highest for the TS group, but not significantly different from control group. Treatments affected NFE digestibility (P = 0.014), and was lowest for the HC group, but only significantly different compared to S, DS and TS treatments, even though the mean ADC in control group was 3.7 % higher than in the HC treatment. DM digestibility was significantly different between groups (P < 0.001) and lower for the HC group compared to other treatments except the LC treatment. Also, the LC treatment resulted in lower ADC for DM compared to the other treatments, however only significantly different from S and TS groups. There were no significant differences in ADCs for phosphorus and ash between treatments. Metabolizable energy was significantly affected by treatments (P = 0.003). Due to the decreased nutrient digestibility, significantly less metabolizable energy was available for the HC group than for the control and sham groups. Furthermore, the energetic cost of growth was significantly different between treatments (P < 0.001), resulting in a significantly higher energy demand for growth of the HC group compared to all other treatments, while the energetic cost of growth for the LC and TS treatments was still significantly higher compared to all other treatments.

Table 1. Effect of experimental conditions on ADC (%) of protein, lipid, NFE, ash, phosphorus and DM, ME and cost of growth during the digestibility trial.

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>S</th>
<th>DS</th>
<th>TS</th>
<th>LC</th>
<th>HC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>91.1</td>
<td>0.16</td>
<td>0.3</td>
<td>0.4</td>
<td>0.25</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td>5 ± a</td>
<td>3 ± a</td>
<td>6 ± b</td>
<td>4 ± a</td>
<td>5 ± c</td>
<td>8 ± c</td>
</tr>
<tr>
<td>Lipid</td>
<td>93.8</td>
<td>0.09</td>
<td>0.07</td>
<td>0.18</td>
<td>0.07</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>8 ± b</td>
<td>5 ± a</td>
<td>3 ± a</td>
<td>9 ± a</td>
<td>7 ± b</td>
<td>6 ± b</td>
</tr>
<tr>
<td>NFE</td>
<td>71.9</td>
<td>0.13</td>
<td>0.16</td>
<td>0.23</td>
<td>1.01</td>
<td>0.43</td>
</tr>
<tr>
<td>Ash</td>
<td>51.8</td>
<td>0.03</td>
<td>0.03</td>
<td>0.04</td>
<td>0.01</td>
<td>0.02</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>7 ± 1.80</td>
<td>5 ± 0.37</td>
<td>2 ± 1.60</td>
<td>7 ± 2.62</td>
<td>8 ± 2.14</td>
<td>9 ± 1.92</td>
</tr>
<tr>
<td>DM</td>
<td>9 ± a</td>
<td>7 ± a</td>
<td>2 ± a</td>
<td>6 ± a</td>
<td>4 ± a</td>
<td>1 ± c</td>
</tr>
<tr>
<td>ME (MJ)</td>
<td>5.38</td>
<td>5.45</td>
<td>5.36</td>
<td>5.34</td>
<td>5.34</td>
<td>5.24</td>
</tr>
<tr>
<td>Cost of growth (kJ g⁻¹)</td>
<td>15.7</td>
<td>0.28</td>
<td>0.56</td>
<td>0.23</td>
<td>0.49</td>
<td>0.41</td>
</tr>
</tbody>
</table>

ADC, apparent digestibility coefficients; NFE, nitrogen free extract including crude fibre (NFE calculated as dry matter – protein – lipid – ash); DM, dry matter; ME, metabolizable energy. Values not sharing a common superscript letter are significantly different. Values are presented as mean ± S.E., n = 3.
3.3. Plasma cortisol

Cortisol implants at two different concentrations led to significantly increased plasma cortisol levels compared to one another and all other treatments (P < 0.001), while there were no significant differences among the control, sham, thermal stress and daily stress treatments. All plasma cortisol results are shown in figure 3. The low cortisol treatment resulted in plasma cortisol levels of 62.2 ± 17.9 ng ml⁻¹, while the high cortisol treated fish had plasma cortisol levels of 152.8 ± 8.9 ng ml⁻¹.

Figure 3. Plasma cortisol levels of the six different treatments after 15 days of the experiment. Values not sharing a common superscript letter are significantly different. All values are presented as means ± S.E., n = 3.

3.4. Organ indices

Organ indices after 15 days of experiment are shown in table 2. Viscerosomatic index was affected by treatment (P < 0.001) and lowest in the HC group showing significant differences to all other treatments. Also HSI was different between groups (P = 0.003), with significant differences between the LC group compared to the TS and HC treatment. The intestinal-somatic index showed significant differences between groups (P = 0.005), resulting in a lower ISI for the HC treatment compared to all other groups except the LC group.

Table 2. HSI, VSI, ISI of the six treatments after 15 days of the experiment.

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>S</th>
<th>DS</th>
<th>TS</th>
<th>LC</th>
<th>HC</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSI</td>
<td>1.25 ± 0.03b</td>
<td>1.29 ± 0.02b</td>
<td>1.25 ± 0.02b</td>
<td>1.03 ± 0.07b</td>
<td>1.45 ± 0.10b</td>
<td>1.15 ± 0.03b</td>
</tr>
<tr>
<td>VSI</td>
<td>14.45 ± 0.40a</td>
<td>14.65 ± 0.36a</td>
<td>13.87 ± 0.02a</td>
<td>13.16 ± 0.83a</td>
<td>13.37 ± 0.51a</td>
<td>9.90 ± 0.88a</td>
</tr>
<tr>
<td>ISI</td>
<td>0.97 ± 0.11a</td>
<td>0.95 ± 0.04a</td>
<td>0.94 ± 0.05a</td>
<td>0.91 ± 0.03a</td>
<td>0.76 ± 0.03ab</td>
<td>0.63 ± 0.03a</td>
</tr>
</tbody>
</table>

HSI, hepatosomatic index; VSI, viscerosomatic index; ISI, intestine somatic index. Values not sharing a common superscript letter are significantly different. All values are presented as means ± S.E., n = 3.

3.5. Lipase activity
The lipase activity in the pyloric caeca, anterior intestine and mesenteric fat at 90 minutes after the last feeding at day 15 of the experiment is shown in figure 4. There were no significant differences in lipase activity among treatments for pyloric caeca or anterior intestine. In the mesenteric fat however, the lipase activity was significantly different between groups (P = 0.003), with higher activity in the HC group compared to the C, S and DS treatments.

Figure 4. Lipase activity in U g⁻¹ wet weight of respective tissue of the six different treatments, 90 minutes after feeding on day 15 of the experiment. Values not sharing a common superscript letter are significantly different. All values are presented as means ± S.E., n = 3.

4. Discussion

The present study demonstrates detrimental effects of chronically elevated levels of plasma cortisol on the performance of rainbow trout through a series of events for which impairment of macronutrient digestion leads to less available metabolizable energy independent of feed intake. Moreover, it is shown that in fish with chronically high cortisol levels there is a decrease in the relative digestive tissue mass, indicating this as one possible cause of impaired digestive capacity. Our findings regarding an increased energetic cost of growth support the role of cortisol as energy mobilizer, not only leading to an impaired energy uptake, but also a higher energy expenditure and consequently a higher cost of growth.

The control and sham groups did not differ in any of the studied parameters, proving that the coconut oil itself seemed to have no effects on the fish, whereas the actions of cortisol showed detrimental effects on the parameters studied. The plasma cortisol levels obtained by the hormone implants were within physiological ranges for rainbow trout facing chronical stressors. For instance, Laidley and Leatherland (1988) reported plasma cortisol levels of ~ 60 ng ml⁻¹ in subordinate rainbow trout due to confinement with a dominant conspecific, while DiBattista (2005) observed plasma cortisol levels of ~ 100 ng ml⁻¹ in a similar experiment. In addition, plasma cortisol concentrations as high as 200 ng ml⁻¹ have been observed for rainbow trout after 48 hours of crowding (Galt et al., 2018).

The fish that were daily stressed by chasing for one minute did not differ from control or sham treatments in any of the studied parameters, indicating that a mild daily stress event of one-
minute duration does not have detrimental effects on rainbow trout. While acute stress affects the integrity of digestive tissues and therefore possibly nutrient uptake, these effects are reversible. Since cortisol elevation and time course in rainbow trout depends on the severity of a stressor (Gesto et al., 2015), we assume that when fish were fed, two hours after being chased with a stick, any elevations in circulating cortisol had returned to baseline levels, not causing any negative effects on digestibility as observed in the cortisol treated fish.

The fish exposed to thermal stress treatment did not show any increase in cortisol levels, suggesting that an elevated temperature kept at 22°C by itself is not a chronic stress factor for rainbow trout, as long as the water quality and oxygen saturation are adequate. However, growth parameters were negatively affected by the elevated temperature, which is more likely explained by the increase in metabolic rate and energy expenditure with a consequently observed higher cost of growth when exposed to elevated temperatures. In fact, raised temperature improved the digestibility of protein and lipid, which is in accordance with former studies in rainbow trout assessing the effect of water temperature on macronutrient digestibility, likely occurring due to elevated digestive enzyme activity (Ng et al., 2003; Yamamoto et al., 2007).

The growth suppressing effects observed in chronically stressed fish due to actions of cortisol are well known and supported by the present study. Interestingly, reduced growth in many previous studies is partly explained by reduced feed intake due to appetite loss in cortisol treated rainbow trout. While in our study, the fish with elevated cortisol levels (63 - 152 ng ml\(^{-1}\)) ate just as well as the control group, chronic plasma cortisol levels of around 70 and 115 ng ml\(^{-1}\) (Madison et al., 2015), or about 200 ng ml\(^{-1}\) (Gregory and Wood, 1999) have been reported to lead to a loss of appetite in juvenile rainbow trout. This difference can possibly be explained by a feeding rate in this study limited to 1.4 % of the tank biomass rather than \textit{ad libitum} feeding in the reported studies. An effect of cortisol on the feed intake would have likely been observed in the current study, if higher feeding rations or \textit{ad libitum} feeding were applied, since cortisol treated fish showed less aggressive feeding behavior and preliminary experiments using \textit{ad libitum} feeding resulted in decreased feed intake of fish treated with cortisol dosages in the same range.

Even though the feed intake of the HC treated fish was not lower than that of the control group, fish only grew approximately half as much. A similar observation was reported by Abbott and Dill (1989), when equal feed rations were consumed by subordinate and dominant rainbow trout,
resulting in higher growth rates of dominant fish, suggesting that factors beyond differences in food intake affect the growth of socially stressed fish. Our results show a dose dependency of cortisol regarding growth rates, since fish cortisol treated groups not only gained significantly less weight than fish of all other groups, but the HC group grew significantly less than the LC group. As a consequence, the FCR was significantly higher for the HC group tested against all other treatments including LC treatment, while the FCR of the LC group was still significantly higher compared to the other four treatment groups. The same trend was observed for the apparent digestibility coefficients, which can partly explain the reduced growth rates observed.

Some previous studies have suggested an impaired digestive performance in cortisol treated and stressed fish, based on observed changes in digestive tissue structure, or high accumulation of fecal matter. To date, no quantitative data to support an effect of cortisol on the digestibility of nutrients have been published. A reduced nutrient digestibility of subordinate Arctic char (Salvelinus alpinus) when compared against dominant fish was observed by Olsen and Ringø (1999), which might have been caused by the chronic stress subordinate salmonids experience due to attacks from their dominant conspecifics. Barton et al. (1987) noted higher accumulations of fecal matter in cortisol treated rainbow trout, and although this was not quantified, they interpreted it as a reflection of decreased absorption capacity. In addition, they observed degenerative changes in the stomach tissue, an observation that was likewise reported in cortisol implanted rainbow trout by Robertson et al. (1963). Hence, impaired absorption of nutrients in previous as well as in the current study could have been caused by changes of the structure of the gastrointestinal tract as reported in former studies. Supportive findings of an effect of stress and possibly cortisol on digestive tissue degeneration were presented by Olsen et al. (2005, 2002), who documented damage to junctional complexes in the midgut and ultrastructural changes of the enterocytes lining the gastrointestinal tract after subjecting rainbow trout and Atlantic salmon (Salmo salar) to acute stress.

Decreased lipid digestibility and reduced available metabolizable energy as observed in the cortisol treated fish in our study has been described in rainbow trout facing a moderate supersaturation in total gas pressure, possibly because of a chronic stressful situation for the fish (Skov et al., 2013). The efficient digestion of lipids requires emulsifiers from endogenous bile acid secretion in the proximal part of the digestive tract (Bakke et al., 2010; Horn, 1997). While
short- and medium-chain fatty acids can be absorbed across the brush border membrane in the anterior intestine via diffusion, bile salts are necessary for emulsifying long chain fatty acids in order to form micelles after cleavage by lipases (Rust, 2002). Therefore, an impaired emulsifying capacity might impede the digestion of released lipids. Changes in bile acid of fish due to stress has previously been reported and may have impaired the lipid digestibility in the current study. Moriarty (1973) noted that stress, and consequently elevated cortisol levels, inhibited the acid secretion in the stomach of Nile tilapia (*Oreochromis niloticus*). The bile secretion from the gall bladder is important for the assimilation efficiency of lipids and therefore bile retention as reported by Earley et al. (2004) could also have impaired the lipid digestibility in the current study. Since lipase plays a central role in the digestion of lipids, we tested if a reduced lipase activity could have caused the decreased digestive efficiency. However, we could not confirm the hypothesis that reduced postprandial enzyme activity in the digestive tissues might negatively affect the nutrient digestibility, since there were no differences in the lipase activity in pyloric caeca or the anterior intestine, where most of the lipids and other nutrients are absorbed in rainbow trout. Contrary to the lipolytic enzyme activity in the digestive tissues, the lipase activity in the mesenteric fat was elevated in the HC treatment demonstrating that cortisol acts as a promoter of energy mobilization from storage tissue, comparable to results by Sheridan (1986), who reported enhanced lipase activity in mesenteric fat in cortisol treated coho salmon.

The observed reduction in intestinal somatic indices in chronically stressed rainbow trout has formerly been reported (DiBattista et al. 2006). In the latter study, ISI of chronically stressed fish were even lower compared to fish that were fasting. A similar finding was reported for the stomach volume of cortisol injected rainbow trout by Høyland (2018), reporting a significantly decreased stomach volume in relation to the body weight. Considering these findings, and those of the current study, a link between elevated cortisol levels and a reduction of digestive tissue mass appears likely. Those observations could explain impaired digestive ability in addition to the structural changes aforementioned, since a decreased volume or weight of digestive tissue also implies a reduced surface for absorption of nutrients.

Due to the impaired digestive efficiency of the HC treated fish, the available metabolizable energy was lower compared to the control and sham group. This, however, can only partly explain the decreased growth rates of cortisol treated fish, since the magnitude of reduction in
available energy (4 %), despite being significantly different, was small compared to the increased energetic cost per unit of growth (83 %) for HC fish compared to the control group. This elevated cost of growth is in line with the general description of cortisol actions explaining reduced growth rates by shifting pathways from energy accretion to energy expenditure to deal with a stressor. Along with the described increased lipolysis in fat reserves, particularly a shift from protein synthesis towards increased protein breakdown in white muscle might be the major factor for reduced growth rates observed in fish with high circulating cortisol levels as this tissue roughly contributes to 50 % of a trout’s body mass. The higher metabolic cost of growth observed in the current study is in agreement with an upregulation of energy-demanding pathways leading to an increased metabolic demand of highly metabolically active tissues such as liver, where elevated gluconeogenic capacity in fish facing high cortisol levels have been described (Mommsen et al., 1999). On a whole body level, this increased energy demand is supported by results from a few previous studies describing increments in basal metabolic rates (Sloman et al., 2000; De Boeck et al., 2001; Lawrence et al., 2019).

5. Conclusion

In summary, the present study demonstrated that chronically elevated cortisol levels negatively influence the performance of rainbow trout by impairing the digestibility of macronutrients, possibly due to an atrophic effect on digestive tissues. Consequently, the available metabolizable energy from a given food ration is lower for fish with high plasma cortisol levels; however, the decrease in available energy alone due to reduced digestive capacity can only explain a small share of the reduced growth observed in cortisol treated fish. A considerable increase in energetic cost of growth due to a shift in energy utilization in cortisol treated fish was conceivably the major cause for the reduced growth of cortisol treated fish. While lipase activity in digestive tissues was not affected by cortisol, the increased lipase activity in the adipose tissue of cortisol treated fish supports the view of the hormone as promoter of energy mobilization from storage tissue.

Credit author statement
### Declaration of interests

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.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Highlights

- Cortisol affects fish performance by impairing digestibility of macronutrients
- Reduced digestibility results in less available metabolizable energy
- Energetic cost of growth is higher in cortisol treated fish
• Elevated plasma cortisol levels lead to atrophy of digestive tissues