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**Keywords**

Eurasian perch, immunity, corticosteroids, proteomics, glucose metabolism.

**Abstract**

The comparative effects of cortisol and 11-deoxycorticosterone (DOC), two major corticosteroids in fish, have yet received little attention in teleosts. We evaluated the proteomic and immune responses of Eurasian perch to chronic corticosteroid treatments. We implanted immature perch with cortisol (80 mg/kg) or DOC (4 mg/kg) and measured the proportions of blood leucocytes, immune indices in the plasma, spleen and liver (complement and lysozyme activity, total immunoglobulin and immune gene expression in the tissues) and differential pro-teome expression (corticosteroid versus control) in the liver and the spleen on days 2, 4 and 14 post-treatment. Implantation of cortisol decreased the ratio of blood leucocytes and depressed Ig levels in both organs while DOC modulated the proportion of leucocyte sub-populations (increase in lymphocytes and decrease in granulocytes). In contrast, the innate humoral immunity was not strongly influenced by any of corticosteroid implants. The only immune parameter that was significantly affected was lysozyme, after DOC treatment. A number of proteins were differentially regulated by these hormones and some were identified in the liver (21 for cortisol and 8 for DOC) and in the spleen (10 for cortisol and 10 for DOC). None of the proteins was directly linked to immunity, except the natural killer enhancing factor, which was repressed by cortisol in the spleen. Our results also confirm that the proteins involved in energetic and glucose metabolism are affected by corticosteroids. Furthermore, these corticosteroids differently regulate immune status in Eurasian perch and they primarily impact leucocytes, as opposed to innate immune function.

## 1. Introduction

Corticosteroids are a vital component of the teleost endocrine system and are involved in the regulation of a range of physiological functions. Corticosteroids can be divided into two groups of hormones: the glucocorticoids, of which cortisol is the primary hormone, and mineralocorticoids such as 11-deoxycorticosterone (DOC), a strong agonist of the mineralocorticoid receptor in teleost fish (Sturm et al., 2005). The effects of these corticosteroid hormones are mediated by intracellular receptors that act as ligand-dependent transcription factors. Under the current paradigm, cortisol binds to either glucocorticoid receptors (GR) or mineralocorticoid receptors (MR) whereas DOC appears to

act only via the MR (Sturm et al., 2005; Arterbery et al., 2011). Whereas cortisol has been shown for a long time to exert pleiotropic actions, the physiological roles of DOC in teleosts are still poorly understood. Still, DOC is often measured at substantial levels in fish notably in Eurasian perch, in the range of some ng/ml (Milla et al., 2009; Mathieu et al., 2013). In particular, the dynamics of plasma DOC during the reproductive cycle suggest its involvement in the final stages of reproduction in a range of fish species (Milla et al., 2009). In trout males, the plasma DOC increase during the spermiation period together with the effects of DOC on spermatocrit values, led to the hypothesis of a role for this hormone in the endocrine control of spermiation in rainbow trout (Milla et al., 2008). But the only confirmed DOC action came out from several studies conducted in the maturing females showing that DOC participates in the triggering of the final oocyte meiotic maturation in some fish species (Nagahama and Yamashita, 2008) while negative effects on sex-steroid secretion have also displayed in both sexes (Milla et al., 2008; Mandiki et al., 2017). Aside from these implications in reproductive functions, other studies suggest its implication in teleost behavior (Takahashi and Sakamoto, 2013) though its role in osmoregulation is still under debate as its capacity to regulate the ionic transporters is species-dependent and generally lower than the cortisol one (McCormick et al., 2008; Kiilerich et al., 2011a, 2011b, 2011c). Finally, a recent study showed up-regulation of plasma DOC level in confined trouts suggesting its implication in fish stress endocrinology (Kiilerich et al., in press). However, as the blood concentration of plasma DOC is 10–1000 fold lower than cortisol depending on the species, the reproductive stage and the stress status (Milla et al., 2009; Kiilerich et al., 2011a, in press), the role of DOC as mineralocorticoid-like hormone acting via the MR is still cryptic. Thus, the research of this hormone in fish raises interesting challenges for fish physiologists. Given the wide ranging effects of cortisol, the identification of specific DOC actions deserves attention.

In vertebrates, including fish, the immune system is regulated, in part, by corticosteroids notably in case of chronic stressor exposure. Indeed, chronic stress, which is often accompanied by elevation of blood cortisol in fish, is generally associated with modulation of hematological and immune parameters (Tort, 2011). For instance, confinement stress induces enhancement of serum cortisol and lysozyme levels, and changes of Reactive Oxygen Species (ROS) production in tilapia Oreochromis mossambicus (Binuramesh et al., 2005) and in Eurasian perch Perca fluviatilis (Douxfils et al., 2011). The immunomodulatory effects of cortisol suggest that these stress effects are partly evoked by this sustained rise of cortisol in the blood. Cortisol is believed to induce a decrease of circulating lymphocyte number, total Ig production and phagocytosis although it may increase the number of phagocytes by limiting neutrophil apoptosis (Harris and Bird, 2000; Esteban et al., 2004). This hormonal immunoregulation appears to be mediated by glucocorticoid receptors (GRs) which are located in the leucocytes and immune organs (Maule and Schreck, 1991; Di Bella et al., 2008; Stolte et al., 2009). Reciprocally, LPS treatment or infection with blood parasites induces variation in GR mRNA expression in the head kidney phagocytes or in the spleen of gilthead seabream, Sparus aurata, common carp, Cyprinus carpio and Eurasian perch Perca fluvia-tilis (Acerete et al., 2007; Stolte et al., 2008; Stolte et al., 2009; Mathieu et al., 2014). While there is a large body of evidence suggesting that cortisol is a potent endocrine regulator of fish immunity, little is known about the influence of other corticosteroids.

Indeed, the role of mineralocorticoids in vertebrate immunity has received very little attention. In mammals, there are scattered reports supporting the participation of aldosterone in immunoregulation. For example, the exposure of human cells to aldosterone induces their adhesion to leucocytes, the synthesis of complement C3, or the activation of CD8(+) T cells (Zach et al., 1993; Krug et al., 2007; Herrada et al., 2010). In rodents, DOC exerts some roles in the inflammatory response in relation with activation of interleukin pathways (Krishnan et al., 2016). In fish, the MR is also expressed in the immune organs, but at lower levels than GRs (Stolte et al., 2009). Aldosterone exerts a suppressive effect on leucocyte phagocytosis in vitro, though the effect is less marked than with cortisol (Law et al., 2001). Recently, we showed that DOC regulates some immune gene expression at short term following a hormonal injection (Mathieu et al., 2013). Thus, we hypothesize that other corticosteroids, such as DOC, participate in hormonal immunoregulation.

In addition to investigation of mRNA and physiological parameters, characterizing the proteome is a useful tool to study the endocrine regulation. Compared to transcriptomics, proteomics is closer to the cellular phenotype and gives a more functional knowledge of the product of gene expression (Silvestre et al., 2012). Notably 2D gel electrophoresis has been used to point out some markers of steroid regulation (Ibarz et al., 2013), stress response (Naderi et al., 2017) or immune response (Hang et al., 2013) in fish. All together, we hypothesize that the immunoregulation of corticosteroids, notably DOC, may be detected at the proteome level using 2D gel electrophoresis.

Our objective was to evaluate the effect of chronic exposure to cortisol and DOC on the immune status of Eurasian perch, an aqua-cultural species known to be quite sensitive to stressors. We injected fish with cocoa butter containing cortisol or DOC and measured blood leucocyte proportions, immune parameters, immune gene expression and the proteomic profile in the liver and spleen. The spleen was selected as it is one of the major lymphoid organ in teleost fishes (Zapata et al., 2006). The liver was selected as this well-known glucocorticoid target contains melanomacrophage centers, antibacterial peptides and high level of immunoglobulins in Eurasian perch (Vijayan et al., 2003; Rossi et al., 2007; Dezfuli et al., 2015).

## 2. Material and Methods

### 2.1. Fish and in vivo corticosteroid implantation

Investigations and animal care were conducted according to the guidelines for the use and care of laboratory animals and in compliance with Belgian and European regulations on animal welfare. One-year-old immature Eurasian perch, Perca fluviatilis (105 ± 15 g) were provided by the CEFRA (Centre de Formation et de Recherches en Aquaculture,

University of Liège, Belgium). Fish were then maintained at the University of Namur (Belgium) experimental fish facilities at 23 °C under constant photoperiod (12 L:12D) at a density of 15 kg/m3 in recirculated water systems. Fish were fed once daily at apparent satiation with a commercial diet and were allowed acclimatizing for 3 weeks before the experiment.

Perch were subjected to corticosteroid administration through the implantation of cortisol (80 mg/kg of fish, Sigma, Steinheim, Germany) or DOC (4 mg/kg of fish, Sigma) or only the cocoa butter vehicle (control) in the body cavity. The DOC concentration was chosen to increase mildly the hormonal level in the plasma to reach supraphysiological concentrations and to avoid pharmacological considerations. Indeed, the average plasma DOC level obtained after DOC implantation (4 mg/kg) corresponds to the highest level measured in one specimen of immature perch (Mathieu et al., 2013; around 10–15 ng/ml). The cortisol implant dose (80 mg/kg) was selected to conduct to cortisol increase in the blood corresponding to the maximum of the plasma cortisol level found in perch specimens after exposure to a sharp stressor (Milla et al., 2010; 650 ng/ml). For both steroids, the choice of these doses allowed to generate a 3–20 fold-increase of the hormone level in the blood relatively to the average level measured in control fish. The difference in the induction level between the 2 steroids, higher for cortisol than DOC, reflects the difference in the stability of their physiological concentrations (high lability and inter-individual variability for cortisol; low variability for DOC).

Implants were prepared by suspending a known concentration of corticosteroids in liquid cocoa butter at 40 °C and by injecting the warm liquid into the peritoneal cavity of the fish (1 ml/100 g. of fish). The cocoa butter solidified rapidly within the fish and acted as a solid implant. Fish sampled before implantation served as initial control. Twelve fish for each treatment (cortisol-treated, DOC-treated and controls) were sampled 2, 4, 14 days after implantation (3 fish per tank at each sampling time, 4 tanks per treatment randomly distributed). The 2 first sampling times were selected as over such middle-term period, some regulations of immune gene expression associated with increase of plasma cortisol and MR gene expression were observed after exposure to handling stress (Milla et al., 2010). The last sampling time was chosen as after 2 weeks of implant exposure, the blood cortisol returns to basal levels. Before sampling, fish were anaesthetized in MS222 (120 mg/l) and blood was collected by caudal vein puncture within 5 min after catching in the tank. Spleen and liver were collected and frozen in liquid nitrogen and stored at −80 °C pending analysis.

### 2.2. Immune parameters, plasma glucose and hemoglobin assays

Lysozyme activity, alternative complement pathway activity (ACH50) and total immunoglobulins were measured after implantation in the plasma, spleen and liver. The protocols have been previously described in the plasma and in the spleen (Milla et al., 2010) while for the liver tissue, we followed the same procedure than that of the spleen. Heamagglutination titre was assayed in the plasma according to (Fatima et al., 2007).

Plasma glycemia was measured as follows. Plasma (50 µl) was de-proteinized by adding 100 µl of perchlorid acid 0.33 mol l− 1 (Hugget and Nixon, 1957) and centrifuged at 3000g (for 10 min at 4 °C). Glucose concentration was determined in the supernatant by the glucose oxidase peroxidase method (Hugget and Nixon, 1957).

### 2.3. ELISA and mass spectrometry for plasma corticosteroids

Plasma cortisol assay was carried out by ELISA (Enzyme-Linked ImmunoAorbent Assay) following manufacturer instructions (BioSource, Nivelles, Belgium) as previously described (Milla et al., 2010). Plasma 11-deoxycorticosterone (DOC) assay was carried out by mass spectrometry as described below. A volume of 100 µl of plasma was homogenized and spiked with 10 µl of a solution of testosterone-D2 (internal standard) at a concentration corresponding to 2 ng µl− 1. Five hundred microliters of water and 2 ml of cyclohexan/ethyl acetate (v/ v) were added. Tube was vortexed for 10 s and centrifuged at 4000 rpm for 15 min. Liquid phase was then transferred to a new tube. Two milliliters of cyclohexan/ethyl acetate (v/v) were added to the solid phase which was again vortexed for 10 s and centrifuged at 4000 rpm for 15 min. Liquid phases were then pooled. Evaporation of the solvent to dryness (SpeedVac) was conducted and followed by the addition of 100 µl of methanol. Finally, the solution was vortexed for 10 s and transferred in an injection vial. Calibration curve (from 0 to 200 ng/ml) was prepared the same way by spiking untreated plasma with a DOC solution prepared in methanol.

A 2690 Alliance Separation Modules (Waters, Milford, MA, USA) integrated autosampler, solvent delivery system and column heater coupled to a Quattro Ultima Platinum triple-quadrupole mass spectrometer (Micromass, Manchester, UK) were used for LC/MS-MS analysis. The LC column used was a Varian Polaris C18 (2 × 150 mm, 3 µm), with a Polaris guard column. The mobile phase was methanol (solvent A) and water containing 0.1% acetic acid (solvent B). The gradient elution conditions were: from 0 to 5 min, from 10% to 100% of solvent A; then, conditions were held 5 min and the contribution of solvent A was decreased to 10% during 2 min. This gradient composition was held 15 min for column reconditioning. The oven temperature was set at 40 °C and the injection volume was 20 µl. The flow rate was 0.25 ml/ min.

The mass spectrometer was equipped with an electrospray ionisation (ESI) interface, used in positive ionisation mode. The MS tune parameters were: capillary: 5 kV, source temperature: 125 °C, desolvation temperature: 250 °C, cone gas flow: 50 l/h, desolvation gas flow: 650 l/h, collision cell pressure: 2 × 10− 3 mbar, multiplier: 650 V. DOC and testosterone-D2 were detected using the Multiple Reaction Monitoring (MRM) mode, with two transitions for each compound (collision energies are given between brackets): DOC: m/z 331 > 97 (20eV) and 331 > 109 (20 eV), testosterone-D2: m/z 291 >111 (20eV) and 291 > 291 (20eV).

Results were calculated using Quanlynx Software (Micromass)

### 2.4. Flow cytometry for differential leucocyte counting

The protocol was adapted from Inoue et al. (2002) except that we were able to separate one unknown population (probably thrombocytes) from lymphocytes. In addition, basophils appeared very rare in Eurasian perch blood. Just before staining, solutions of DiOC6(3) (3,3 dihexyloxacarbocyanine, Sigma, Steinheim, Germany) at 500 µg ml− 1 of ethanol were diluted 10 times in Hank's Balanced Salt Solution (HBSS, Sigma, Steinheim, Germany) just before staining. Ten microliters of fresh blood were dispensed into a test tube in presence of 1950 µl of HBSS and 40 µl of DiOC6. The tubes were mixed gently and incubated at room temperature for 10 min. After staining, blood cells were analyzed using FACS (Fluorescence-Activated Cell Sorting, FAC-Scalibur, Becton Dickinson, Erembodegem, Belgium). Forward scatter (FSC), side scatter (SSC) and green fluorescence (FL-1) of each cell were measured. In a FL-1 vs SSC dot-plot, the red blood cells were separated from the white blood cells as demonstrated by Inoue et al. (2002) and the ratio number of leucocytes over the number of red blood cells was calculated. After leucocytes separation in a FSC vs SSC dot-plot, the different leucocyte populations were identified and their relative proportion (number of leucocytes of the population/total number of leucocytes) was calculated using the software Cell Quest ProTM (Fig. 1). The categories of leucocyte populations were the following: thrombocytes, lymphocytes, monocytes and the combination of neutrophils and eosinophils, these two latter populations being impossible to split.

### 2.5. Total RNA extraction, reverse transcription and Real-time PCR

RNA extraction, reverse transcription and Quantitative Real-time PCR (QPCR) procedures were carried out on spleen and liver as described previously by (Milla et al., 2006). Real-time PCR analysis was performed using a SYBR Green PCR Master Mix (Applied Biosystem, Warrington, UK) using 600 nmol l− 1 of primers. Amplification parameters were as follows: each of the 40 cycles consisted in 15 s of denaturation at 95 °C, 1 min annealing/extension at 56–61 °C depending on gene. Actin mRNA abundance was determined for use as an internal standard because of it stability during the experiment for all treatments. Primer sequences, melting temperatures and amplicon size are described by in Table 1. In addition to classical immune genes regulated by corticosteroids, the MR was chosen as the potential receptor for both corticosteroids and the GR2 as another potential receptor for cortisol.

### 2.6. Proteomic analyses

Proteins from spleen and liver tissue were extracted from fish exposed to cortisol and 11-deoxycorticosterone after 14 days of exposure. There were six replicates per treatment. One unit of spleen was homogenized with 5 units (10 units for liver) of DLA buffer (30 mmol l− 1 Tris/HCl, 4% w/v CHAPS, 2 mmol l− 1 thiourate, 7 mol l− 1 urea, pH 8.5) containing protease inhibitor cocktail (Sigma). The soluble protein fractions were harvested by centrifugation at 13000× rpm for 15 min and the pellet discarded. Supernatants were aliquoted into 0.5 ml ependorff microcentrifuge tubes and protein concentration was determined using the method of Bradford with BSA as a standard (1 g/l, Sigma). A fixed amount of 220 µg of proteins was taken from each sample, mixed in rehydration buffer to a volume of 220 µl to which was previously added 3 mg/ml of DTT and 5 µl/ml of IPG buffer 4–7 (GE healthcare, Uppsala, Sweden) and then was loaded onto 11 cm IPG strips (linear pH 4–7, GE healthcare). Rehydration was performed passively overnight at room temperature. The first-dimensional IEF was performed at 20 °C with the following parameters: 500 V for 1 h, 1000 V for 1 h (gradient), 6000 V for 2 h30 (gradient) and 3000 Vh. After equilibration in a buffer containing 6 M urea, 50 mM Tris (pH 8.8), 30% (v/v) glycerol, 2% SDS and 1% blue bromophenol, with 65 mM DTT for 15 min and subsequently with 260 mM iodoacetamide for another 15 min, a bidimensional system (Criterion Biorad, Hercules, CA) filled with 10% precast acrylamide gels (Biorad) was used for the second dimensional separation. Electrophoresis was performed at 160 V for 10 min and at 200 V until bromophenol blue line had reached the bottom of the gels. The resolved 2D spots were stained with Coomassie Brilliant Blue G250 (Biorad). Gels were scanned with an Imagemaster scanner and all images were analyzed by ImageMaster 2D Platinum 6.0 software (GE Healthcare). Parameters used for spot detection were minimal area = 5 pixels, smooth factor = 2 and saliency = 12–18. The gel chosen as the reference had the best percentage of match with the other gels (from 65 to 78%). The reference gel was then used for matching of corresponding protein spots between gels. Following average mode of background subtraction, individual spot intensity volume was normalized with total intensity volume (summation of the intensity volumes obtained from all spots in the same 2-DE gel). The normalized intensity volume values of individual protein spots were then used to determine differential protein expression between control and corticosteroid-treated groups. Multiple t-tests were performed to determine differentially expressed proteins.

### 2.7. Mass spectrometry (MS) for protein identification

Some differently expressed spots were manually excised from preparative gels and proteins were digested with trypsin by in-gel digestion. The choice of the proteins spots was made on the basis of their: (i) good gel resolution (for efficient hand excision); (ii) normalized volume, which must reach a threshold and presence on more gels than the half of the total number of gels. The gel pieces were twice washed with distilled water and then shrunk with 100% acetonitrile. The proteolytic digestion was performed by the addition of 3 µl of modified trypsin (Promega) suspended in 0.1 mol l− 1 NH4HCO3 cold buffer. Proteolysis was performed overnight at 37 °C. The supernatant was collected and combined with the eluate of a subsequent elution step with 5% v/v formic acid. The eluates were kept at −20 °C prior to analysis. The digests were separated by reverse phase liquid chromatography using a 75 µm × 150 mm reverse phase NanoEase Column (Waters) in a CapLC (Waters, USA) liquid chromatography system. Mobile phase A was 95% of 0.1% formic acid in water and 5% acetonitrile. Mobile phase B was 0.1% formic acid in acetonitrile. The digest (15 µl) was injected and the organic content of the mobile phase was increased linearly from 5% B to 40% in 40 min and from 40% B to 100% B in 5 min. The column effluent was connected to a PicoTip emitter (New Objective) inside the Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) source. Peptides were analyzed in the DDA mode on a Q-TOF2 (Waters)

instrument. In survey scan, MS spectra were acquired for 1 s in the m/z range between 450 and 1500. When intensity of 2+ or 3+ ions increased above 20 counts/s there was an automatic switch to the MS/MS mode. The collision-induced dissociation (CID) energy was automatically set according to mass to charge (m/z) ratio and charge state of the precursor ion. Acquisition in MS/MS was stopped when intensity fell below 5 counts/s or after 15 s. Q-TOF2 and CapLC were piloted by MassLynx 4.0 (Waters). For the electrospray survey, background was subtracted with a threshold of 35%, polynomial order 5. For smoothing, we used the Savitzky-Golay method with 2 iterations and a window of 3 channels. Finally, we assigned the mass of peaks with 3% of threshold, a minimum peak width of 4 channels and a centroid top method at 80%. For MS/MS raw data, we performed a rigorous deisotoping method with a threshold of 3%. Peak lists were created using ProteinLynx Global Server 2.2.5 (Waters) and saved as PKL file for use with Mascot 2.2 (Matrix Science). Enzyme specificity was set to trypsin, and the maximum number of missed cleavages per peptide was set at one. Carbamidomethylation was allowed as fixed modification and oxidation of methionine as variable modification. Mass tolerance for mono-isotopic peptide window and MS/MS tolerance window were set to ± 0.3 Da. Scaffold (version Scaffold-2_06_01, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.2) and X! Tandem (The GPM, thegpm.org; version 2007.01.01.). Peptide identifications were accepted if they could be established at > 95% probability as specified by the Peptide Prophet algorithm (Keller et al., 2002). Protein identifications were accepted if they could be established at > 99% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm. This whole procedure was applied twice in the liver to ensure a good identification of the proteins and to check the repeatability of this identification.

### 2.8. Statistical analysis (except proteomics)

The changes in physiological and immune parameters were analyzed by using a two-way analysis of variance (ANOVA, sometimes carried out on log or arcsine square root transformed data for percentage values) followed by a post-hoc LSD test to determine significant differences between sampling time and/or between corticosteroid-treated and control fish. The fish was used as the experimental unit. Kolmogorov-Smirnov test was used to evaluate the normality of the data while homogeneity of variances was assessed by Bartlett test. The level of significance was set at 5%.

## 3. Results

### 3.1. Steroid release

Plasma cortisol levels were significantly higher in the cortisol-implanted fish (700 ng/ml) compared with the control group (22 ng/ml; $p < 10^{-4}$, Table 2). Cortisol levels peaked 2 days post-treatment (1064 ng/ml) in the cortisol implant group then stabilized at ~300 ng/ ml between days 4 and 14, but were always significantly higher than in the control fish ($p < 10^{-4}$). In addition, we detected a significant increase in DOC release throughout the experiment ($p < 10^{-4}$). There was a significant difference between control and DOC-treated fish on days 4 ($p < 0.05$) and 14 ($p < 10^{-2}$).

### 3.2. Effect of corticosteroids on blood glucose and hematological indices

Both corticosteroid treatments induced significant changes in blood glucose (Table 3). The mean concentration was significantly lower in control fish (32.6 mg/dl) than the mean concentrations in cortisol-(89.3 mg/dl; time *treatment interaction; $p < 10^{-4}$) and DOC-implanted fish (38.7 mg/dl; treatment effect without time *treatment interaction; $p < 0.05$). The only differences between treated and control fish at each time point were between cortisol-treated fish and the control group on days 2 ($p < 10^{-4}$) and 4 ($p < 10^{-3}$).

Neither hematocrit nor hemoglobin values were significantly affected by corticosteroid implants (Table 3).

### 3.3. Effect of corticosteroids on complement / lysozyme activity and gene expression

Corticosteroid implantation did not have any significant effect on the plasma immune parameters (complement activity, haemagglutination titre, lysozyme activity and total immunoglobulin) (Table 3). However, we did observe an effect of these treatments in the liver and spleen (Table 4). Implantation with cortisol increased splenic ACH50 throughout the entire sampling period (treatment effect; $p < 0.05$), but the effect was only significant on day 14 (time *treatment interaction; $p < 0.05$). Liver lysozyme activity was higher in DOC-treated fish than in controls on day 14 (time *treatment interaction; $p < 0.05$). Splenic lysozyme activity was also influenced by the interaction between sampling time and DOC treatment ($p < 0.01$), and levels were lower in DOC-treated fish on day 2 ($p < 0.05$). We detected an effect similar to this interaction on spleen lysozyme mRNA expression, with significantly lower levels on day 4 ($p < 0.05$). Last, cortisol treatment was associated with a decrease in the quantity of total immunoglobulins in the liver on day 2 (time *treatment interaction; $p < 0.05$) and in the spleen on day 14 (time *treatment interaction; $p < 0.01$).

### 3.4. Effect of corticosteroids on immune and corticosteroid receptor gene expression

Corticosteroid treatments had no effect on the expression of selected immune genes in the liver or the spleen while the expression was variable on time for some genes (Table 5). In the spleen, the interaction treatment *sampling time had a significant effect on MR gene expression in cortisol-treated fish ($p < 0.01$). The level was higher on day 2 in cortisol-implanted fish ($p = 0.05$) but lower on day 14 ($p < 0.01$). In both organs, the GR2 gene expression level remained unaffected (data not shown).

### 3.5. Effect of corticosteroids on blood cell proportions

The ratio of leucocytes to erythrocytes (WBC/RBC) decreased significantly in cortisol-, but not DOC-, implanted fish when compared with control fish (treatment effect; $p < 0.05$, Fig. 2A). Cortisol also induced a significant decrease in the percentage of the undetermined population over the course of the experiment (treatment effect; $p < 0.01$, Fig. 2B). DOC treatment had no effect on the percentage of this population but was associated with a significant increase in the percentage of lymphocytes on day 2 (time *treatment interaction; $p < 0.05$, Fig. 2C). Conversely, the percentage of eosinophil/neutrophil cells was significantly lower in DOC-treated fish (25.4%) when compared with control fish (34.8%, treatment effect; $p < 0.01$) without significant time *treatment interaction (Fig. 2D). Last, corticosteroid treatment had no effect on the percentage of monocytes, though we observed a high level of variability among individuals (Fig. 2E).

### 3.6. Effect of corticosteroids on hepatic and splenic proteins

The expression patterns of perch liver and spleen proteomes are illustrated in Fig. 3. The average number of spots (for: pI 4–7 and molecular weight 15–150 kDa) was 846 in the liver and 625 in the spleen. After 14 days exposure to corticosteroids, the number of differentially expressed protein spots in the liver was 60 between cortisol and control groups ($p < 0.01$) and 35 between DOC and control groups ($p < 0.02$). We chose 23 and 13 spots, respectively, for identification by Q-TOF. In the spleen, the number of differentially expressed protein spots was 49 between the cortisol and control groups ($p < 0.02$) and 61 between the DOC and control groups ($p < 0.02$). We chose 19 and 22 spots, respectively, for identification by Q-TOF. The properties of the proteins analyzed by Q-TOF are summarized in Tables 6–9. We identified 21 and 8 differentially regulated proteins in the liver following treatment with cortisol and DOC, respectively (Tables 6 and 7). These proteins mainly participate to the krebs cycle, the glucose metabolism (glycolysis, neoglucogenesis), the cellular metabolism and two chaperones were detected. We identified 10 differently regulated proteins in the spleen in both the cortisol and DOC treatment groups (Tables 8 and 9). The only protein directly involved in the immune response was the natural killer enhancing factor which was repressed in the spleen after the cortisol treatment. The same classes of functional groups appeared for both organs (cellular and glucose metabolism, chaperone), and at least one structural protein (actin, tubulin or keratin) was regulated in each group.

## 4. Discussion

In fish, at least two corticosteroids appear to be of physiological importance. The first, cortisol, is well-known and exerts pleiotropic actions that affect osmoregulation, immunity, energetic metabolism and reproduction. The second, 11-deoxycorticosterone (DOC), is a putative ligand of the mineralocorticoid receptor whose function has only recently been evaluated. Our DOC measurements in control fish provide estimates of baseline DOC levels in immature Eurasian perch, (2–4 ng/ ml) and suggest a low inter-individual variability. Consistent with previous observations (Noaksson et al., 2005), our results suggest that 11-deoxycorticosteroids are present in higher levels in non-maturing, unstressed perch than in their salmonid counterparts (Doyon et al., 2006; Milla et al., 2009). It is unknown whether this difference reflects inter-family specializations in the functions of 11-deoxycorticosteroids.

The corticosteroid implants used in our study induced supraphysiological cortisol level at day 2 and then prolonged hormone elevation at days 4 and 14 at average level corresponding to the maximum of the physiological concentration found in Eurasian perch for both steroids (Milla et al., 2010; Mathieu et al., 2013). However, the changes in plasma DOC are poorly understood so we have few bases for determining if these supraphysiological levels observed in our study are biologically relevant within the context of specific physiological situations. Notably, there is no indication today that DOC is involved or not in the stress response in fish. Given the potency of DOC to activate the mineralocorticoid receptor (MR) in fish (e.g. Sturm et al., 2005), we speculate that the physiological actions of DOC are mediated by MR activation. The regulation of MR gene expression by cortisol (Milla et al., 2010; present study) suggests that MR also regulates cortisol receptivity in Eurasian perch. However, this regulation shows a dual pattern, with initial increase of MR gene expression followed by decrease after 14 days. At short term, this data confirms that cortisol positively regulates the splenic MR gene expression in Eurasian perch (Milla et al., 2010; Mathieu et al., 2013) and at long term, it may support that the drop of corticosteroid receptor gene expression could limit the adverse consequences of long-term cortisol exposure. Together with the induction of Heat Shock Proteins (HSP 60, HSP 70 and HSP 90) in this study, actors of the corticosteroid receptor transduction pathways, we hypothesize involvement of these receptors in the following corticosteroid actions. However, the circulating cortisol and DOC levels are physiologically high and chronic maintenance of such high levels may have triggered downstream effects. For instance consistently high levels of cortisol down-regulates glucocorticoid receptors even if it was not observed at the transcript level for GR2. Itis thus difficult to know if the observed effects are due to direct corticosteroid actions via binding to their receptors or to indirect feedback effects of corticosteroids after target tissue desensitization/alteration. The use of specific antagonists of corticosteroid receptors would have been useful to address this question.

### 4.1. Corticosteroid treatment affects the abundance of proteins involved in glycolysis, neoglucogenesis and krebs cycle

Both corticosteroid treatments induced an increase in blood glucose. The hyperglycemic effect of cortisol has also been observed in other fish species following cortisol implantation (e.g. Vijayan et al., 2003) and the role of cortisol in glucose metabolism is well-known (Faught and Vijayan, 2016). The changes of protein levels involved in glycolysis (enolase 1, pyruvate kinase and glyceraldehyde 3-phosphate dehydrogenase) and the neoglucogenetic enzyme pyruvate carboxylase is consistent with the observed hyperglycemia. As an aside, we also confirmed that cortisol modulates glycemia by regulating the quantity of enzymes involved in glycolysis. We also noted that some of the proteins involved in the Krebs cycle were down-regulated by cortisol (fumarase, fumarate hydratase, malate dehydrogenase and

its precursor) illustrating the progressive weakening of energetic metabolism during prolonged exposure to cortisol.

To our knowledge, this is the second report of an effect of DOC on blood glucose in fish (Ng and Woo, 1990). In amphibians, aldosterone treatment may promote an increase of blood glucose (Herman, 1992). In mammals, MR is involved in the regulation of several enzymes linked to glucose homeostasis, including glucose-6-phosphatase, phosphoenolpyruvate carboxykinase and fructose-1-6-biphosphatase 1 (Liu et al., 2006). Indirectly, an excess of aldosterone can lead to hypokalemia and, in turn, prevent insulin secretion (Calle et al., 2003). For the first time, our results suggest that a number of DOC-regulated proteins play a role in glucose metabolism (e.g., glyceraldehyde-3-phosphate dehydrogenase, fumarate hydratase and pyruvate kinase). Thus, we hypothesize that the DOC/MR system is involved in glycemia maintenance in Eurasian perch and, probably, in other fish species. However, given the low levels of plasma DOC and the lower efficacy at inducing hyperglycemia, we hypothesize that this mineralocorticoid-like hormone complements the action of glucocorticoids.

### 4.2. Leucocytes proportion and immunoglobulin production: a target for chronic cortisol exposure

The proportion of leucocytes relative to red blood cells decreased in the blood following treatment with cortisol. Because hematocrit and hemoglobin values were not affected, we hypothesize that this decrease is due to leucocyte suppression, not polycythemia. A decrease in leucocyte numbers is common in fish following stress (e.g. Rotllant et al., 1997). In a recent study, treatment with the GR agonist dexamethasone caused a decrease in the relative percentage of leucocytes in common carp (Balabanova et al., 2009). According to Dhabhar (2002), this phenomenon is not due to leucocyte lysis but rather to cell redistribution in lymphoid organs. In accordance with this hypothesis, we observed an increase in spleen Ig after handling stress in Eurasian perch (Milla et al., 2010). In vitro, cortisol had no effect on splenic Ig levels both after 2 days of culture (Milla et al., 2010) and in vivo within 4 days (this study). Interestingly though, the levels in the spleen decreased 14 days after cortisol implantation. Thus, we believe that cortisol is not a short-term regulator of splenic leucocyte/Ig incorporation in Eurasian perch, though chronic exposure to cortisol may have an effect. The effect of cortisol was observed earlier in the liver (day 2) than in the spleen suggesting there is a differential time-course in the sensitivity of the organs to Ig suppression following chronic cortisol elevation. Last, cortisol influenced the abundance of natural killer enhancing factor in the spleen, a potential stimulator of cytotoxic activity differently regulated after bacterial infection (Chen et al., 2009). Taken together, our data would indicate that, as in other species, cortisol affects leu-cocyte/Ig quantity and activity in Eurasian perch. In previous studies, the effects of cortisol on leucocytes have been proved to be mediated through glucocorticoid receptors (Maule and Schreck, 1991; Stolte et al., 2009). But this GR involvement remains to be demonstrated in Eurasian perch.

### 4.3. Differential effects of corticosteroids on leucocyte sub-populations

Despite the putative effect on whole leucocyte quantity, cortisol did not bias the proportion of leucocytes toward lymphocytes or granulocytes. In common carp, Cyprinus carpio, activated B-lymphocytes underwent apoptosis in the presence of cortisol in vitro while the neutrophilic granulocytes were rescued from apoptosis. In addition, cortisol elevation stress-induced or injection-induced was associated with a drop in circulating lymphocytes in common carp and in Eurasian perch (Engelsma et al., 2003; Mathieu et al., 2013). In salmonids, exposure to stress as well as long-term cortisol exposure promotes a drop in blood lymphocytes (Barton et al., 1987; Wiik et al., 1989; Ruane et al., 2000). In our study, the inability of cortisol to induce changes in the relative proportion of leucocyte sub-populations might be due to the insensitivity of leucocyte apoptosis to the present kinetics of cortisol release, to the route of cortisol exposure, or to the short sampling period (2 weeks).

In contrast, a significant decrease in the percentage of neutrophils was observed following DOC treatment. Our proteomic approach did not highlight markers of granulocyte functioning that were regulated by DOC, with the exception of transferrin, an iron binding protein involved in granulocyte maturation and during inflammation in mammals (Evans et al., 1986; Gorbunov et al., 2005). In fish, through the binding and transporting of iron, transferrin is thought to participate in immune regulation, antimicrobial and antioxidant activity, cytoprotection and electron transport (Stafford and Belosevic, 2003; Ong et al., 2006) and there are growing reports supporting its immunomodulatory role. For example, in roughskin sculpin Trachidermus fasciatus, transferrin gene expression was up-regulated in immune organs during a LPS treatment and exposure to heavy metals (Yingying et al., 2012). In mammals, aldosterone promotes a decrease in the number of neutrophils (Miller et al., 1994) and was recently shown to inhibit nuclear factor κB activation in neutrophils, thus acting as an anti-inflammatory inducer (Bergmann et al., 2010) In Eurasian perch, an acute elevation of blood DOC after injection did not modify the proportion of neutrophils (Mathieu et al., 2013). While data linking mineralocorticoids and granulocytes are scarce, it is tempting to speculate that chronic DOC elevation may exert a detrimental effect on granulocyte functioning and that mineralocorticoids may play a role in regulating neutrophil levels and activity in vertebrates. The DOC effect on the increase of lymphocyte percentage was transient and still supports that the profile of leucocytes is modulated at short term. Although data linking mineralocorticoids and lymphocytes does not exist in fish, the current mammalian literature indicates that aldosterone would regulate the activation of lymphocytes (Amador et al., 2014). Further studies are needed to test this hypothesis in fish and the molecular events involved. More generally, our results may also be in favor of a significant impact that corticosteroids have on cellular immunity in Eurasian perch even if providing data on the absolute levels of leucocyte sub-populations rather than relative ones would have also allowed to be more conclusive about the effects on leucocyte production.

### 4.4. Corticosteroids do not appear as strong regulators of innate immune proteins

We did not detect a change in plasma immune parameters following corticosteroid treatment. Thus, our current data

support that plasma immune markers are resistant to strong and chronic corticosteroid elevation (some hundreds of ng/ml) in Eurasian perch. In the organs, cortisol had no effect on gene expression, protein quantity, or the activity of proteins linked to innate immunity despite a surprising increase in complement activity in the spleen on day 14. Therefore, chronic exposure to cortisol exposure (2 weeks) does not appear to affect the innate immunity of Eurasian perch. However, many immune proteins, potentially corticosteroid-responsive, are not detected by such proteomic approach, such as those expressed in small amounts or those quickly released after hormonal treatment. The choice of other technical conditions (e.g. gel features) or other characteristics of the experimental set up (sampling times) might have conducted to the highlight of relevant immune candidates. In addition, we notice a high variability in the measurement of some immune parameters following these corticosteroid treatments. Thus, we cannot rule out the fact that a lower variability or the test of other parameters would have allowed to detect more corticosteroid effects.

After DOC treatment, the only effect was a change in lysozyme activity and/or lysozyme gene expression in both the liver and spleen. We previously reported DOC effect on lysozyme gene expression in the spleen but the kinetics of hormone release is not comparable (Mathieu et al., 2013). However, we notice the absence of correlation between lysozyme mRNA abundance and lysozyme activity, which has ever been observed in fish (e.g. Massart et al., 2014) and might reflect post transcriptional and post-translational changes. The regulation of lysozyme in the liver and spleen has been reported following challenge tests with pathogens or LPS exposure in fish (Saurabh et al., 2011; Hang et al., 2013), this may support the role of tissular lysozyme in the fish immune response.

Some other proteins have also been shown to be regulated by DOC even if their role in fish immunity remains a matter of investigation. Catalase, which was up-regulated in the liver, has often been shown to be induced after challenge tests with pathogens (Dautremepuits et al., 2003; Xu et al., 2017). It is known that the antioxidant enzymes such as catalase are enable to scavenge reactive oxygen species thereby protecting cells from oxidative damage. Induction in the hepatic activity of this antioxidant enzyme in relation to cellular stress has been proposed to counteract putative infection (Dautremepuits et al., 2003; Kumar et al., 2017). Cell Division protein 48 (Cdc48), known as p97 or valosin-containing protein (VCP) in mammals, is an abundant AAA-ATPase that is essential for many ubiquitin-dependent processes. One well-documented role for Cdc48 is in facilitating the delivery of ubiquitylated misfolded endoplasmic reticulum proteins to the proteasome for degradation (Gallagher et al., 2014). The ubiquitin-proteasome system (UPS) components plays a central role in the generation of epitopes presented by MHC class I molecules at the cell surface for immune recognition. For instance, within the context of tumor diseases, Cdc48 p97/VCP alteration is linked to MHC class I presentation of some antigens (Ebstein et al., 2016). Interestingly, Cdc 48 has rarely been investigated in fish biology and one of the scarce studies showed downregulation of this protein in the teleost fish Paralichthys olivaceus after challenge with Streptococcus parauberis (Cha et al., 2012). His role for the maintenance of cellular function and the recovery of cellular damage after bacterial challenge in fish would deserve some investigations. Protein disulfide isomerases (PDIs) is another DOC-regulated protein which is thought to contribute to protein folding and assembly in the endoplasmic reticulum. Growing evidence including in fish suggests that PDIs is involved in host cell invasion and that they are relevant targets for the host immune response after pathogen infection (Sha et al., 2012). In mammals, evidence is growing that MR signaling regulates basal monocyte/macrophage function (Rickard et al., 2009). However, our study does not provide evidence that chronic exposure to mild levels of mineralocorticoids potently modulate non-specific immunity.

In conclusion, we suggested that chronic exposure to both cortisol and DOC may affect glucose metabolism in Eurasian perch. Both corticosteroids induced distinct effects on immunity. Cortisol affected the ratio of leucocytes and the level of tissue Ig. DOC influenced the proportion of leucocyte sub-populations, lysozyme activity in the organs and few proteins whose immune function remains to be validated or demonstrated in teleost fish species (transferrin, catalase, cdc48 and Protein Disulfide Isomerase). Therefore, while leucocytes appear to be primary targets of chronic exposure to corticosteroids, they are not, chronically, strong regulators of innate immune proteins in Eurasian perch.

**Table 1. Sequences and melting temperature (Tm) of primers used for gene expression quantification.**

| Primers | Sequence (5′-3′) | Tm (°C) | Amplicon size |
|---|---|---|---|
| Actin forward (Q-PCR) | ACCTTCTACAACGAGCTGAGAGT | 60.4 | 153 |
| Actin forward (Q-PCR) | AGTGGTACGACCAGAGGCATA | 60.2 | |
| pfGR2 forward (Q-PCR) | GCAACGGCAACATGCTCTGCTTCGCA | 65.9 | 142 |
| pfGR2 reverse (Q-PCR) | TCGTCGTTGGAAACCTGCAGCCGGA | 65.8 | |
| pfMR forward (Q-PCR) | AGCGCTGCCAATGCCCTGGT | 64.5 | 198 |
| pfMR reverse (Q-PCR) | TGCTTTCCGGCCAGGCGGTTGA | 65.5 | |
| Lysozyme forward (Q-PCR) | TGACTGGGTTTGTCTGAGCAAGTG | 60.1 | 212 |
| Lysozyme reverse (Q-PCR) | GATGCCATAGTCAGTGGATCCGTC | 60 | |
| Hepcidin forward (Q-PCR) | CAGCCACTGAAGTGCAAGAGCTG | 56.5 | 154 |
| Hepcidin reverse (Q-PCR) | GCGACACTTAAAGCCACGCTTGTG | 54.2 | |
| C3 forward (Q-PCR) | GTACCAGCTCTTTGGGTGTCAGCA | 61.8 | 134 |
| C3 reverse (Q-PCR) | GTAAGCCCTCATGTCCCATAGCAG | 62 | |
| Apolipoprotein A1 forward (Q-PCR) | GTCGAGGACATCAAGACTCAGCTC | 59.7 | 145 |
| Apolipoprotein A1 reverse (Q-PCR) | TAGGACTGACAGGTAGGTTAGGTG | 57.8 | |

**Table 2. Effects of cortisol and 11-deoxycorticosterone (DOC) on plasma concentration after steroid implantation in Eurasian perch. Means ± SD (n = 4–6). •indicates significant differences between control and corticosteroid-implanted fish (2-way ANOVA and LSD tests, p < 0.05).**

| | Treatment | D2 | D4 | D14 |
|---|---|---|---|---|
| Plasma cortisol (ng/ml) | Control | 17 ± 10 | 18 ± 11 | 15 ± 8 |
| | Cortisol | 1064 ± 784• | 311 ± 159• | 260 ± 112• |
| | DOC | 19 ± 16 | 41 ± 29 | 47 ± 15 |
| Plasma DOC (ng/ml) | Control | 2.9 ± 0.5 | 4.6 ± 1.5 | 3 ± 0.4 |
| | Cortisol | 2.7 ± 0.7 | 2.9 ± 1.7 | 2.8 ± 0.5 |
| | DOC | 6.5 ± 2.5 | 15.1 ± 8.5• | 24.2 ± 15.1• |

**Table 3. Effects of cortisol and 11-deoxycorticosterone (DOC) on plasma physiological and im-mune parameters after steroid implantation in Eurasian perch. Means ± SD (n = 9–12). Different letters indicate significant interaction treatment *time effect (2-way ANOVA and LSD tests, p < 0.05). □: significant treatment effect of the hormone without significant interaction effect.**

| | Treatment | D2 | D4 | D14 |
|---|---|---|---|---|
| Glucose (mg/dl) | Control | 34 ± 7a | 36 ± 13a | 28 ± 7a |
| | Cortisol | 139 ± 98c | 78 ± 29b | 54 ± 19ab |
| | DOC | 37 ± 12□ | 45 ± 18□ | 34 ± 14□ |
| Hematocrit (%) | Control | 28 ± 3 | 29 ± 7 | 21 ± 2 |
| | Cortisol | 28 ± 6 | 31 ± 5 | 27 ± 2 |
| | DOC | 25±3 | 28±5 | 25±9 |
| Hemoglobin (mmol/dl) | Control | 2.4 ± 0.8 | 3 ± 0.8 | 2.2 ± 0.9 |
| | Cortisol | 2.3 ± 0.5 | 2.7 ± 0.6 | 2 ± 0.3 |
| | DOC | 2.3 ± 0.4 | 3.1 ± 1.2 | 2.5 ± 0.4 |
| Complement activity (U/ml) | Control | 510 ± 179 | 546 ± 136 | 405 ± 147 |
| | Cortisol | 462 ± 179 | 405 ± 161 | 437 ± 175 |
| | DOC | 535 ± 228 | 489 ± 198 | 454 ± 108 |
| Haemagglutination titre (inverse dilution) | Control | 22.6 ± 10.6 | 22.9 ± 10.7 | 43.6 ± 23.6 |
| | Cortisol | 39.6 ± 16.6 | 20.7 ± 11.5 | 25.4 ± 14.5 |
| | DOC | 29.5±19 | 24.5±15.6 | 21.1 ±11.9 |
| Lysozyme activity (U/ml) | Control | 131 ± 51 | 196 ± 37 | 147 ± 64 |
| | Cortisol | 127 ± 65 | 117 ± 33 | 144 ± 68 |
| | DOC | 137 ± 31 | 161 ± 53 | 117 ± 51 |
| Immunoglobulin (µg/µl) | Control | 3.8 ± 3.8 | 2.4 ± 1.6 | 2 ± 2.4 |
| | Cortisol | 1.8 ± 1.8 | 2.8 ± 2.4 | 2.6 ± 3 |
| | DOC | 2.4 ± 3.2 | 2.4 ± 2.4 | 1.4 ± 1.8 |

**Table 4. Effects of cortisol and 11-deoxycorticosterone (DOC) on total Ig, complement activity and C3 gene expression, lysozyme activity and gene expression, in spleen and liver after steroidimplantation in Eurasian perch.**

**Means ± SD (n = 6). Different letters indicate significant interaction treatment *time effect (2-way ANOVA and LSD tests, p < 0.05).**

| | Treatment | D0 | D2 | D4 | D14 |
|---|---|---|---|---|---|
| Liver complement activity (ACH50) | Control | 14.7 ± 4.3 | 18.5 ± 7.2 | 12.7 ± 3.4 | 14.6 ± 7.8 |
| | Cortisol | | 12.3 ± 4.4 | 19.4 ± 14.5 | 10 ± 3.2 |
| | DOC | | 15.6±4.2 | 11.4±3 | 13.9±2.6 |
| Liver C3 mRNA (relative abundance) | Control | 0.9 ± 0.4 | 1.1 ± 0.5 | 1.1 ± 0.1 | 1 ± 0.7 |
| | Cortisol | | 0.5 ± 0.3 | 0.8 ± 0.5 | 1.2 ± 0.9 |
| | DOC | | 1.3 ± 0.6 | 0.7 ± 0.5 | 1 ± 0.7 |
| Spleen complement activity (ACH50) | Control | 38.1 ± 9.7 | 42.5 ± 9.4ab | 32.9 ± 2.1a | 33.7 ± 5a |
| | Cortisol | | 56.2 ± 4.4b | 44.7 ± 14.5ab | 52.2 ± 3.2b |
| | DOC | | 41.1 ±9.8 | 43±11.5 | 42.3±16 |
| Spleen C3 mRNA (relative abundance) | Control | 0.7 ± 0.3 | 0.9 ± 0.7 | 1.6 ± 1 | 1.1 ± 0.5 |
| | Cortisol | | 1.2 ± 0.6 | 1.3 ± 0.8 | 0.7 ± 0.4 |
| | DOC | | 0.9 ± 0.4 | 1.1 ± 0.6 | 1.3 ± 0.8 |
| Liver lysozyme activity (U/ml) | Control | 103 ± 40 | 127 ± 43ab | 120 ± 27ab | 94 ± 20a |
| | Cortisol | | 96 ± 20 | 82 ± 29 | 137 ± 87 |
| | DOC | | 101 ± 18ab | 103 ± 21ab | 138 ± 34b |
| Liver lysozyme mRNA (relative abundance) | Control | 0.7 ± 0.3 | 0.7 ± 0.4 | 1 ± 0.6 | 1.1 ± 1 |
| | Cortisol | | 0.9 ± 0.4 | 0.8 ± 0.6 | 1.2 ± 0.4 |
| | DOC | | 1±0.5 | 1±0.7 | 1.1±1 |
| Spleen lysozyme activity (U/ml) | Control | 169 ± 69 | 992 ± 432b | 1055 ± 334b | 777 ± 207ab |
| | Cortisol | | 1061 ± 338 | 1165 ± 547 | 915 ± 175 |
| | DOC | | 607 ± 339a | 814 ± 384ab | 1134 ± 350b |
| Spleen lysozyme mRNA (relative abundance) | Control | 1.7 ± 1.3 | 1 ± 1.3a | 3.1 ± 1.8b | 2.1 ± 2ab |
| | Cortisol | | 2.1 ± 1.3 | 2.1 ± 0.7 | 2 ± 1.5 |
| | DOC | | 0.7±0.6a | 1.8±1.1a | 1.4±0.7a |
| Liver immunoglobulin (µg per 5 µl of buffer) | Control | 1.7 ± 0.7 | 1.9 ± 0.6b | 1.6 ± 0.8b | 2.1 ± 0.4b |
| | Cortisol | | 1 ± 0.5a | 2.4 ± 1b | 2.3 ± 0.7b |
| | DOC | | 2 ± 0.9 | 1.8 ± 0.6 | 1.7 ± 0.9 |
| Spleen immunoglobulin (µg per 5 µl of buffer) | Control | 0.5 ± 0.3 | 0.5 ± 0.4b | 0.5 ± 0.1b | 0.6 ± 0.4b |
| | Cortisol | | 0.5 ± 0.2b | 0.3 ± 0.2ab | 0.2 ± 0.2a |
| | DOC | | 0.7 ± 0.2 | 0.5 ± 0.4 | 0.4 ± 0.4 |

**Table 5. Effects of cortisol and 11-deoxycorticosterone (DOC) on other immune gene expression after steroid implantation in Eurasian perch. Means ± SD (n = 6). Different letters indicate significant interaction treatment *time effect (2-way ANOVA and LSD tests, p < 0.05).**

| | Treatment | D0 | D2 | D4 | D14 |
|---|---|---|---|---|---|
| Liver Apolipoprotein A1 mRNA (relative abundance) | Control | 1.6 ± 0.9 | 2.1 ± 1.2 | 2.4 ± 1.1 | 1.8 ± 0.6 |
| | Cortisol | | 1.9 ± 1.4 | 2.6 ± 1.1 | 2.1 ± 0.9 |
| | DOC | | 1.8 ± 0.6 | 1.9 ± 1.2 | 1.9 ± 0.8 |
| Liver hepcidin mRNA (relative abundance) | Control | 1.5 ± 0.7 | 1.3 ± 0.5 | 1.7 ± 0.8 | 1.5 ± 0.5 |
| | Cortisol | | 1.8 ± 0.6 | 1.7 ± 0.4 | 1.3 ± 0.6 |
| | DOC | | 1.1± 0.3 | 1.9 ± 0.8 | 1.2 ± 0.9 |
| Liver MR mRNA (relative abundance) | Control | 0.8 ± 0.4 | 0.5 ± 0.2 | 1.3 ± 0.6 | 1.2 ± 0.7 |
| | Cortisol | | 0.5 ± 0.2 | 1 ± 0.4 | 1.4 ± 1.1 |
| | DOC | | 0.6 ± 0.5 | 1.4 ± 0.6 | 0.7 ± 0.4 |
| Spleen Apolipoprotein A1 mRNA (relative abundance) | Control | 1.7 ± 0.6 | 1.8 ± 1 | 2.2 ± 1.1 | 2.5 ± 1.6 |
| | Cortisol | | 2.2 ± 2 | 3.6 ± 3 | 1.8 ± 0.9 |
| | DOC | | 1.2 ± 0.6 | 1.8 ± 1.2 | 1.6 ± 0.8 |
| Spleen hepcidin mRNA (relative abundance) | Control | 1.4 ± 0.7 | 1.7 ± 0.9 | 2.1 ± 1.1 | 1.9 ± 2.1 |
| | Cortisol | | 1.2 ± 1.1 | 2.3 ± 1 | 2.1 ± 0.8 |
| | DOC | | 1.6 ± 0.6 | 1.4 ± 0.8 | 1.3 ± 0.9 |
| Spleen MR mRNA (relative abundance) | Control | 1 ± 0.4 | 0.4 ± 0.3a | 2.7 ± 1.3c | 2.1 ± 1.4bc |
| | Cortisol | | 1. ± 0.7b | 2.6 ± 1.3c | 0.5 ± 0.4a |
| | DOC | | 0.5 ± 0.3 | 1.4 ± 0.7 | 1.3 ± 0.8 |

**Table 6. Detailed list of protein identified by nano LC-MS-MS differently expressed in liver of Perca fluviatilis following cortisol implantation.**

| Spot number | Accession number | Protein name | Species | Matching peptides | Theorical pI/Mw (kDa) | Fold change treated/control |
|---|---|---|---|---|---|---|
| Krebs cycle | | | | | | |
| 3093 | Q7SX99 | Fumarate hydratase, mitochondrial | Danio rerio | 5 | 9.7/55 | –8.3 |
| 3097 | Q4RUH5 | Fumarate hydratase | Danio rerio | 6 | 9.7/49 | –5 |
| 3244 | B5DGS4 | Malate dehydrogenase | Salmo salar | 9 | 8.8/35 | –3.4 |
| 3309 | C1BLX1 | Malate dehydrogenase mitochondrial precursor | Osmerus mordax | 9 | 8.1/35 | –2 |
| Glucose metabolism | | | | | | |
| 3292 | Q9DDG7 | Enolase | Salmo trutta | 2 | 5.3/40 | Infinite |
| 3303 | C0HBL8 | Pyruvate kinase | Salmo salar | 3 | 5.4/57 | –1.5 |
| 3270 | C1BIY8 | Glyoxylate reductase/hydroxypyruvate reductase | Osmerus mordax | 4 | 8.5/39 | –2.6 |
| 3319 | A5H1I2 | Glucose-regulated protein 94 | Hippoglossus olivaceus | 3 | 4.7/92 | –5 |
| 3291 | Q8AYN3 | Pyruvate carboxylase | Pagrus major | 10 | 5.6/113 | 2.1 |
| 2981 | Q8AXQ6 | Pyruvate carboxylase | Pagrus major | 5 | 5.9/113 | –4.3 |
| Cellular metabolism | | | | | | |
| 3345 | Q2PZI2 | Gamma-aminobutyrate aminotransferase | Carassius auratus | 3 | 5.7/29 | 2.1 |
| 3045 | B5X2T3 | Aldehyde dehydrogenase | Salmo salar | 12 | 5.8/57 | –1.5 |
| 3519 | Q98TM9 | putative aldehyde dehydrogenase | Platichthys flesus | 2 | 5.2/14 | –3.2 |
| iG3110 | B5X2T3 | Aldehyde dehydrogenase, mitochondrial precursor | Salmo salar | 7 | 5.8/59 | –1.3 |
| 3383 | C3KID8 | Sulfotransferase 1C1 | Anoplopoma fimbria | 4 | 5.6/36 | –3.1 |
| 3491 | Q4SVN9 | Serine hydroxymethyltransferase | Tetraodon nigroviridis | 6 | 6.6/53 | –2.6 |
| 3458 | ACO09314 | Delta3,5åelta2,4åienoyl-CoA isomerase, mitochondrial precursor | Osmerus mordax | 2 | 6.65/33 | Infinite |
| 3379 | B5X0X8 | Agmatinase, mitochondrial precursor | Salmo salar | 5 | 6.1/40 | –1.5 |
| Chaperone | | | | | | |
| 3168 | Q596W9 | HSP 70 | Rhabdosargus sarba | 9 | 5/71 | 2.12 |
| Other protein functions | | | | | | |
| 3545 | Q4SVD2 | Chromosome undetermined SCAF13763, whole genome shotgun sequence | Tetraodon nigroviridis | 3 | 4.8/28 | –2.9 |
| 3014 | Q4RMX3 | Tubulin alpha chain | Esox lucius | 8 | 50/4.8 | 0.28 |

**Table 7. Detailed list of protein identified by nano LC-MS-MS differently expressed in liver of Perca fluviatilis following DOC implantation.**

| Spot number | Accession number | Protein name | Species | Matching peptides | Theorical pI/Mw (kDa) | Fold change treated/control |
|---|---|---|---|---|---|---|
| Krebs cycle | | | | | | |
| 1453 | C1BLX1 | Malate dehydrogenase mitochondrial precursor | *Osmerus mordax* | 9 | 8.1/35 | 2.2 |
| 1273 | Q7SX99 | Fumarate hydratase, mitochondrial | *Danio rerio* | 5 | 9.7/55 | 3.4 |
| Glucose metabolism | | | | | | |
| 997 | Q8AYN3 | Pyruvate carboxylase | *Pagrus major* | 7 | 6.1/129 | 2.5 |
| Cellular metabolism | | | | | | |
| 1677 | Q4SVN9 | Serine hydroxymethyltransferase | *Tetraodon nigroviridis* | 2 | 6.6/53 | 3.7 |
| 1063 | Q5XW25 | Catalase | *Oplegnathus fasciatus* | 3 | 9.1/60 | 3.2 |
| Chaperone | | | | | | |
| 1051 | Q4S9T9 | HSP60 | *Tetraodon nigroviridis* | 10 | 5.6/65 | 3 |
| Other protein functions | | | | | | |
| 1643 | Q4SVD2 | Eukaryotic translation initiation factor 3 | *Tetraodon nigroviridis* | 2 | 5.2/30 | 2.6 |
| 1385 | Q802U0 | Beta actin | *Dicentrarchus labrax* | 3 | 5.2/42 | 2.6 |

**Table 8. Detailed list of protein identified by nano LC-MS-MS differently expressed in spleen of Perca fluviatilis following cortisol implantation.**

| Spot number | Accession number | Protein name | Species | Matching Peptides | Theorical pI/Mw (kDa) | Fold change Treated/ Control |
|---|---|---|---|---|---|---|
| Glucose metabolism | | | | | | |
| 554 | B5AAJ6 | Glyceraldehyde 3-phosphate dehydrogenase isoform 2 | *Oplegnathus fasciatus* | 5 | 6.2/47 | − 1.9 |
| 545 | B5AAJ6 | Glyceraldehyde 3-phosphate dehydrogenase isoform 2 | *Oplegnathus fasciatus* | 5 | 6.4/36 | − 2.1 |
| 360 | Q6IQP5 | Enolase 1, alpha | *Danio rerio* | 8 | 6.2/47 | − 1.6 |
| 350 | B5X1B5 | Alpha-enolase | *Salmo salar* | 5 | 5.5/47 | − 2.6 |
| Cellular metabolism | | | | | | |
| 298 | B5X2T3 | Aldehyde dehydrogenase mitochondrial precursor | *Salmo salar* | 5 | 5.9/57 | − 1.3 |
| Chaperone | | | | | | |
| 505 | D9ZNB4 | Heat Shock Protein 90 | *Lutjanus sanguineus* | 3 | 5.4/83 | 1.62 |
| Immunity | | | | | | |
| 757 | Q1KLP9 | Natural killer enhancing factor | *Psetta maxima* | 4 | 5.5/22 | − 1.5 |
| Other protein functions | | | | | | |
| 520 | Q91219 | Simple typeII keratin K8B | *Oncorhynchus mykiss* | 4 | 5/59 | − 3.8 |
| 512 | A4L7J0 | Beta actin | *Thunnus maccooyii* | 3 | 5.5/37 | 4 |
| 631 | H9ETZ1 | Protein SET | *Macaca mulatta* | 2 | 5/34 | − 2.3 |

**Table 9. Detailed list of protein identified by nano LC-MS-MS differently expressed in spleen of Perca fluviatilis following DOC implantation.**

| Spot number | Accession number | Protein name | Species | Matching peptides | Theorical pI/Mw (kDa) | Fold change treated/ control |
|---|---|---|---|---|---|---|
| Glucose metabolism | | | | | | |
| 1183 | Q8JJC2 | Pyruvate kinase | *Takifugu rubripes* | 2 | 4.9/58 | − 1.9 |
| 1406 | B5AAJ6 | Glyceraldehyde-3-phosphate dehydrogenase isoform 2 | *Oplegnathus fasciatus* | 2 | 6.2/36 | − 2.3 |
| Cellular metabolism | | | | | | |
| 1006 | B9V308 | Transferrin | *Epinephelus coioides* | 3 | 5.9/33 | − 8.3 |
| 997 | B9V308 | Transferrin | *Epinephelus coioides* | 3 | 5.9/33 | − 1.4 |
| 1471 | B8XQT3 | CdC48 | *Larimichthys crocea* | 2 | 5.2/89 | − 6.2 |
| Chaperone | | | | | | |
| 1215 | B0S564 | Protein disulfide isomerase | *Danio rerio* | 4 | 4.6/57 | − 1.3 |
| Other protein functions | | | | | | |
| 1476 | AF186109 | TPM4 ALK fusion oncoprotein | *Homo sapiens* | 2 | 4.2/28 | 1.51 |
| 889 | Q4RQP0 | Chromosome 2 SCAF15004, whole genome shotgun sequence | *Tetraodon nigroviridis* | 2 | 4.7/92 | − 1.9 |
| 1429 | A4L7J0 | Beta actin | *Thunnus maccoyii* | 3 | 5.5/37 | 1.83 |
| 1545 | A4L7J0 | Beta actin | *Thunnus maccooyii* | 3 | 5.5/36 | − 5 |

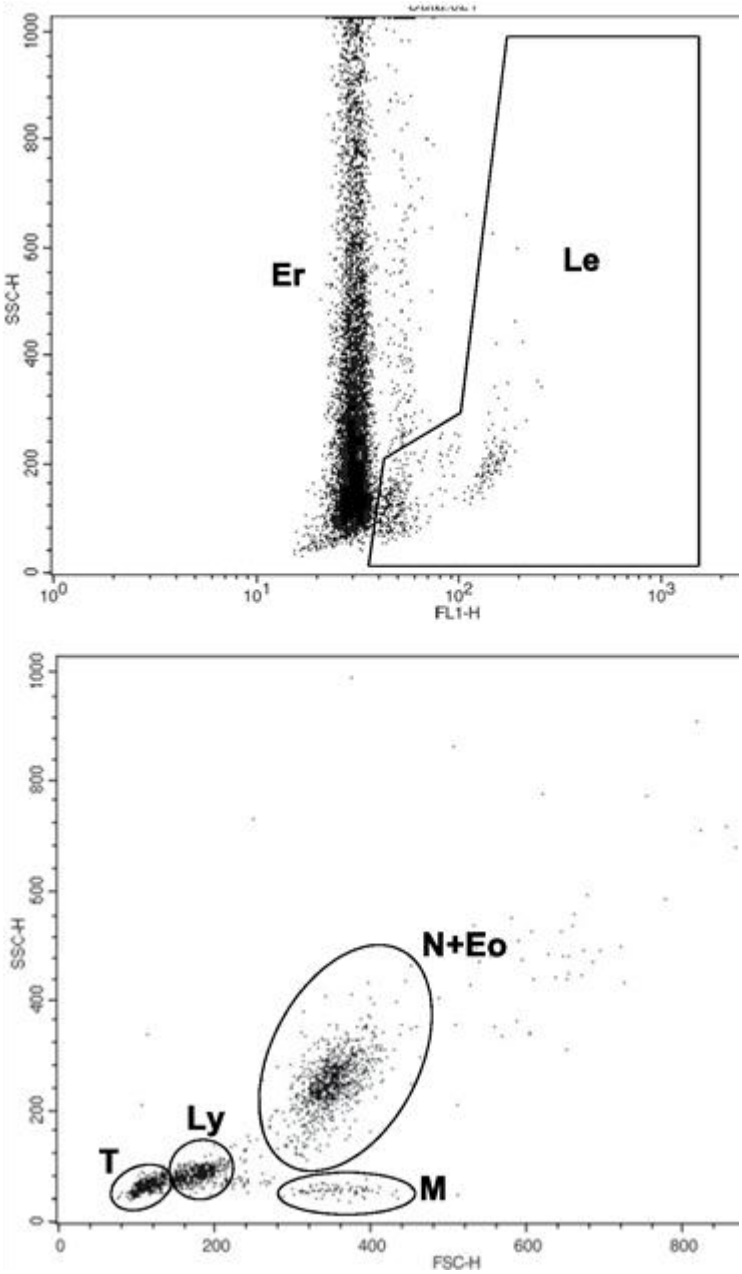


**Figure 1.**
FACS analysis of Eurasian perch blood cells stained with DiOC6. The plot SSC vs FL1 (A) reveals 2 distinct cell populations: the erythrocytes (Er) and the leucocytes (L). The plot SSC vs FSC (B) reveals 4 distinct leucocyte populations: the undetermined population (T), the lymphocytes (Ly), the neutrophil and eosinophil granulocytes together (N + Eo) and the monocytes (M).

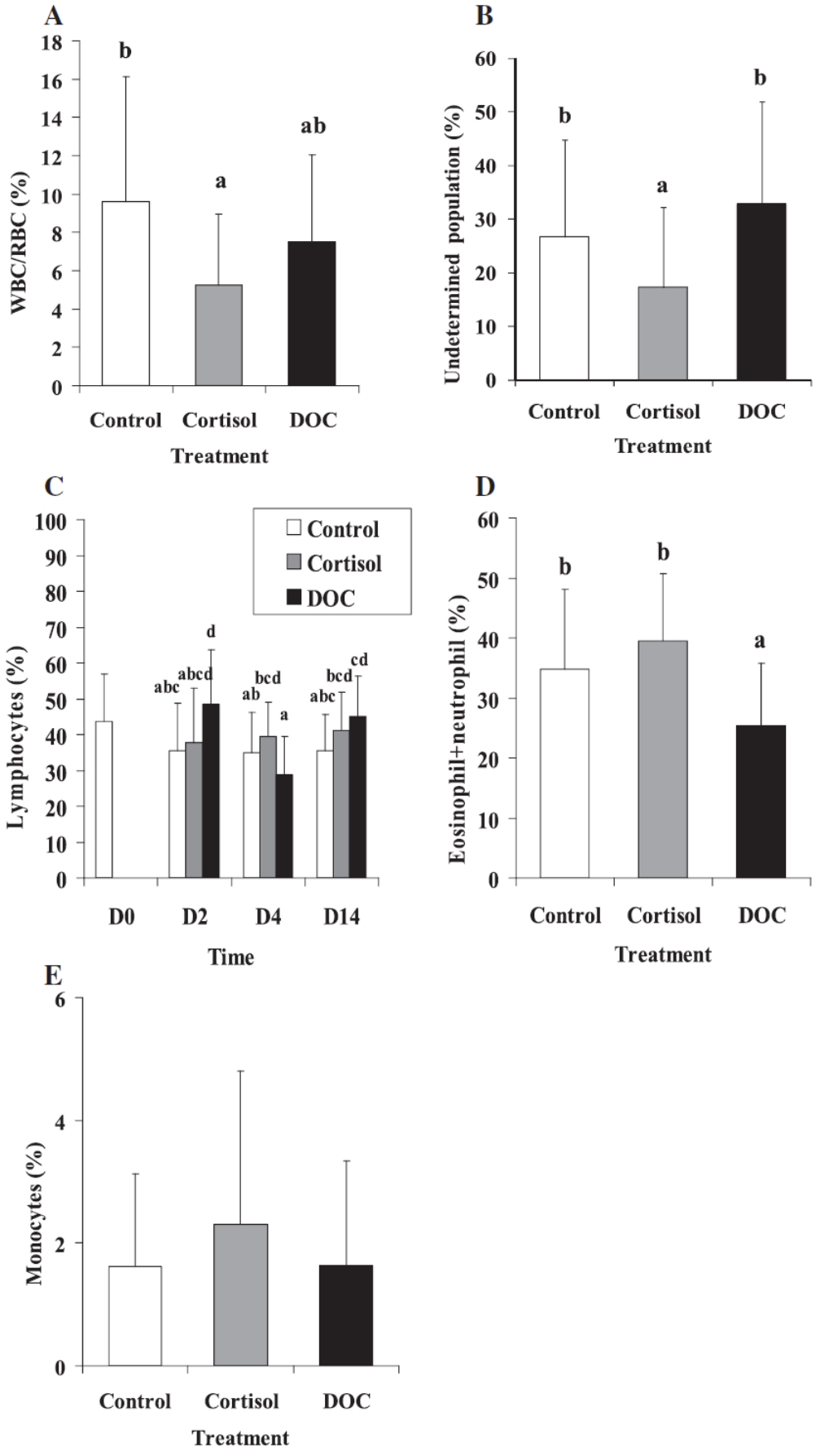


**Figure 2.**

Effects of cortisol and 11-deoxycorticosterone (DOC) on the proportion of leucocyte sub-populations after steroid implantation in Eurasian perch according to the sampling time and/or the hormonal treatment. (A) White blood cells/red blood cells, (B) undetermined population (%), (C) lymphocytes, (D) eosinophil + neutrophile leucocytes, (E) monocytes. Values are mean + SD (N = 6–33). Different letters indicate significant differences between treatments (2-way ANOVA and LSD tests, $p < 0.05$).

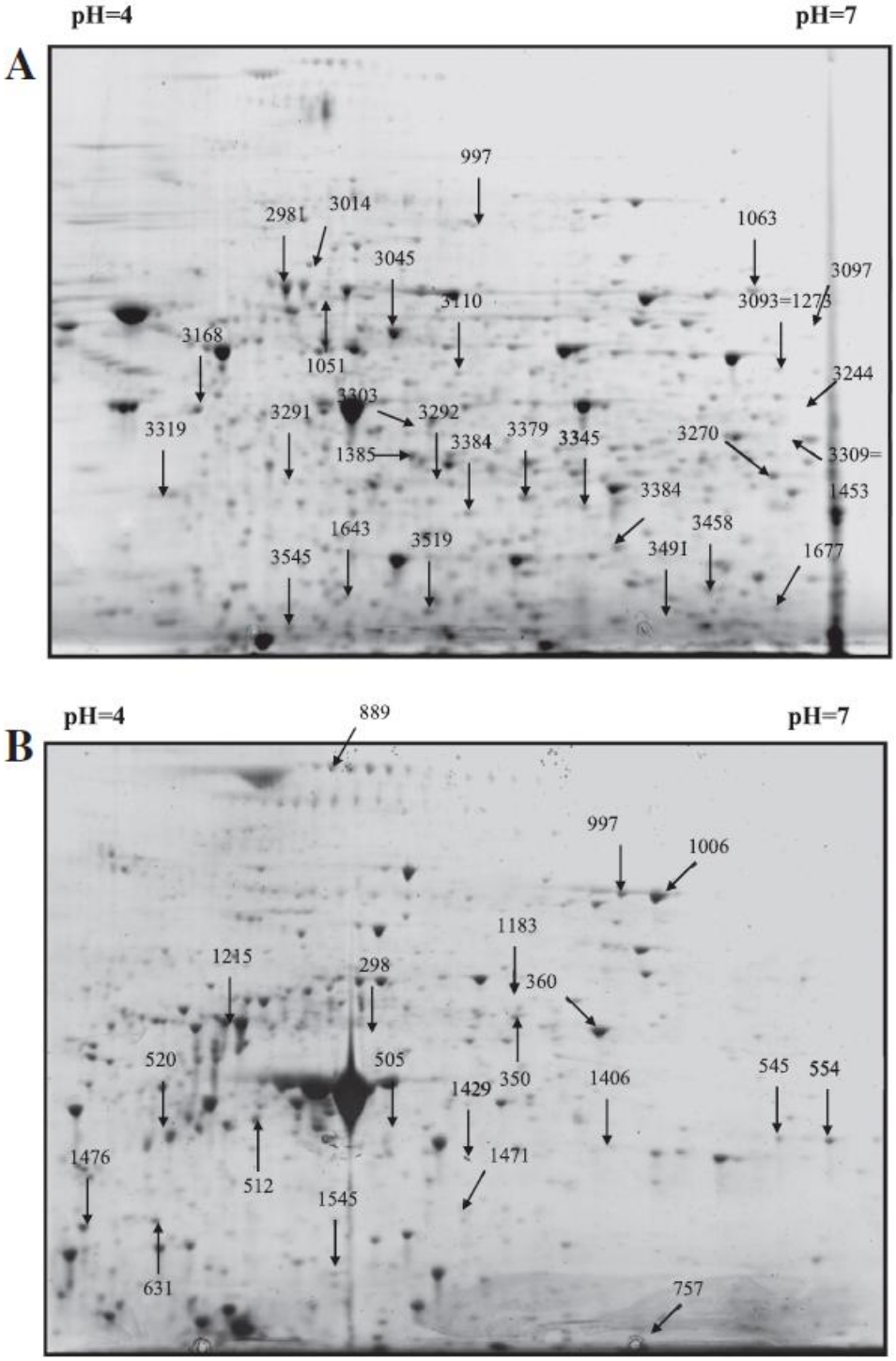


**Figure 3.**

Proteome of Eurasian perch liver (A) and spleen (B) using a two-dimensional PAGE. Gels were done with 220 µg of total protein extract loaded into IPG strips pH 4–7. After migration, gels were stained with G250 coomassie blue. The labelled spots, differently expressed between control fish and corticosteroid treated fish, are listed in Tables 6–9.

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