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# Effects of stress and cortisol on the polarization of carp macrophages

Magdalena Maciuszek[a], Leszek Rydz[a], Iga Świtakowska[a], B.M. Lidy Verburg-van Kemenade[b], Magdalena Chadzińska[a,*]

[a] Department of Evolutionary Immunology, Institute of Zoology and Biomedical Research, Faculty of Biology, Jagiellonian University, Gronostajowa 9, PL30-387, Krakow, Poland

[b] Cell Biology and Immunology Group, Wageningen University, P.O. Box 338, 6700AH, Wageningen, the Netherlands

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ABSTRACT

In teleost fish, myelopoiesis is maintained both in the head (HK) and trunk kidney (TK), but only the HK holds the endocrine cells that produce the stress hormone cortisol. We now compared the effects of prolonged restraint stress (*in vivo)* and cortisol (*in vitro*) on the polarization of HK and TK-derived carp macrophages.

Monocytes/macrophages from both sources were treated *in vitro* with cortisol, lipopolysaccharide or with both factors combined. *In vivo,* fish were challenged by a prolonged restraint stress. Gene expression of several markers typical for classical M1 and alternative M2 macrophage polarization, as well as glucocorticoid receptors, were measured.

Cells from both sources did not differ in the constitutive gene expression of glucocorticoid receptors, whereas they significantly differed in their response to cortisol and stress. In the LPS-stimulated HK monocytes/macrophages, cortisol *in vitro* counteracted the action of LPS while the effects of cortisol on the activity of TK monocytes/macrophages were less explicit.

*In vivo*, restraint stress up-regulated gene expression of M2 markers in freshly isolated HK monocytes/macrophages, while at the same time it did not affect TK monocytes/macrophages. Moreover, LPS-stimulated HK monocytes/macrophages from stressed animals showed only minor differences in the gene expression of M1 and M2 markers, compared to LPS-treated monocytes/macrophages from control fish. In contrast, stress-induced changes in TK-derived LPS-treated cells were more pronounced. However, these changes did not clearly indicate whether in TK monocytes/macrophages stress will stimulate classical or alternative polarization.

Altogether, our results imply that cortisol *in vitro* and stress *in vivo* direct HK, but not TK, monocytes/macrophages to the path of alternative polarization. These findings reveal that like in mammals, also in fish the glucocorticoids form important stimulators of alternative macrophage polarization.

## 1. Introduction

Macrophages are crucial cells for both innate and adaptive immune responses. They are extremely flexible and, depending on the cytokine microenvironment and the presence of pathogens, they can polarize towards classically polarized cells (M1 macrophages) and alternatively polarized (M2 macrophages) [1,2]. In mammals, the polarization of macrophages towards M1 occurs under the influence of tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), granulocyte-macrophage colony-stimulating factor (GM-CSF), lipopolysaccharide (LPS) and lipoteichoic acid (LTA). M1 polarized macrophages synthesize pro-inflammatory cytokines (including IL-1, IL-12), chemokines (CXCL8-11), reactive oxygen species (ROS) and nitric oxide (NO). In this way, a pro-inflammatory cascade is started, leading to elimination of the pathogen [1,3].

Alternatively-activated M2 (regulatory, anti-inflammatory) macrophages are more heterogeneous than M1 cells. It has been proposed [3–5] that different M2 phenotypes (M2a, M2b, or M2c) participate in diverse activities, all aimed at suppression and regulation of the inflammatory response to avoid host tissue damage. Upon stimulation with IL-4 and IL-13, M2a cells increase their arginase activity and thus promote killing of parasites. M2b macrophages are induced by immune complexes, by TLR stimulation or by antagonizing the response of the IL-1 receptor with the receptor antagonist (IL-1ra). These cells thus exert immune regulatory functions. Finally, IL-10- or glucocorticoid-stimulated M2c macrophages suppress the immune response and promote tissue remodeling [6]. Another classification of alternatively-activated macrophages was proposed by Mosser and Edwards [7]. They suggested the existence of two populations of M2 cells: wound healing

* Corresponding author.
*E-mail address:* magdalena.chadzinska@uj.edu.pl (M. Chadzińska).

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cells and immune regulatory cells. Wound healing macrophages arise during a Th2-driven immune response or during an innate immune response driven by basophilic granulocytes and play a crucial role in tissue repair. Regulatory macrophages arise upon stimulation with immune complexes, G-protein coupled receptor ligands, glucocorticoids or IL-10 and exert anti-inflammatory activities due to their capacity to down-regulate IL-12 and to enhance the production of IL-10.

Despite the phenotypic differences, all M2 macrophages are characterized by an immunosuppressive action leading to silencing of inflammation, wound healing and return of the tissue to its state before infection/damage. M2 macrophages produce large amounts of anti-inflammatory cytokines such as IL-10, IL-4, IL-13 or transforming growth factor β (TGF-β) and they are characterized by a high activity of arginase 1 and high expression of the IL-4 receptor (IL-4R), mannose receptor (MR, CD206) and Fizz1 molecules [8]. Moreover, M1 and M2 macrophages clearly differ in the intracellular metabolism of L-arginine. In M1 there is an increase in the expression of the inducible nitric oxide synthase (iNOS), converting L-arginine to $N^6$-hydroxy-L-arginine, then to citrulline and consequently to increased production of nitric oxide. In M2, however, there is an increased expression of arginase converting L-arginine to urea and L-ornithine, and then to proline and polyamines (putrescine, spermidine, and spermine). In this way, cell proliferation, production of collagen and other components promoting tissue repair and fibrosis are increased [9].

Evidence is accruing that fish macrophages polarize similarly to their mammalian counterparts, as their stimulation with LPS or LPS with IFN-γ, induces expression of typical M1 polarization markers: pro-inflammatory cytokines, CXC chemokines, iNOS and release of NO [10], while after *in vitro* stimulation with cAMP, carp macrophages express markers of alternative (M2) polarization: arginase 2 and arginase activity [11]. It was also found that recombinant IL-4/13A and IL-4/13B significantly decrease production of ROS and NO. Moreover, it down-regulates expression of IL-1β, TNF-α and iNOS, but increases mRNA expression of the anti-inflammatory cytokines (TGF-β and vascular endothelial growth factor - VEGF)) and arginase-2 in the head kidney monocytes/macrophages from the large yellow croaker [12]. Similarly, Hodgkinson et al. [13] found that goldfish recombinant IL-4/13A and IL-4/13B increased both the expression and activity of arginase and down-regulated the IFNγ induced production of NO. Finally, Nguyen-Chi et al. [14] found that, in response to wounding of zebrafish, unpolarized macrophages are recruited to the site of inflammation and adopt a M1-like phenotype. Subsequently, they progressively convert their functional phenotype from M1-like to M2-like in response to the progressive change in the inflammatory microenvironment within the tissue.

As mentioned before, in mammals, glucocorticoids induce polarization of M2c or regulatory macrophages. An anti-inflammatory macrophage phenotype was also promoted by annexin 1 (ANXA1, also known as lipocortin I) [15]. Annexin 1 is a glucocorticoid-induced protein and like glucocorticoids, both ANXA1 and N-terminal peptides derived from the parent molecule inhibit eicosanoid synthesis, block leukocyte migration and induce apoptosis of inflammatory cells. Moreover, in macrophages they stimulate the release of IL-10, while they inhibit the expression of iNOS and IL-12, and the release of NO [16].

Although the effects of stress and cortisol on the immune response of teleost fish has been studied intensively (for review see Verburg-van Kemenade et al. [17]), little is known about their involvement in the polarization of fish macrophages. Fish are interesting animals to study such interactions as their main myelopoietic organ - head kidney - is also the equivalent of the mammalian adrenal glands where stress hormones (cortisol and catecholamines) are produced in interrenal and chromafine cells, respectively [17]. This suggests that the maturation environment of monocytes/macrophages in this site can be under the direct paracrine action of stress hormones. In contrast, myelopoiesis and maturation of macrophages in the trunk kidney is not directly affected by stress hormones.

Therefore, we now aim to compare the effect of stress, cortisol and N-terminal ANXA1-derived peptide annexin 1-(2–26) (glucocorticoid-regulated protein) on the pro- and anti-inflammatory activity/M1 and M2 polarization of macrophages derived from the head and the trunk kidney of carp. *In vitro*, the effect of cortisol was compared while *in vivo* we studied the effect of restraining stress on the expression of genes that are putative markers for M1 and M2 polarization in teleost fish.

## 2. Materials and methods

### 2.1. Animals

Young individuals of common carp (*Cyprinus carpio*; body weight 40–60 g, 6–8 months; line R3xR8) were obtained from the Institute of Ichthiobiology and Aquaculture, Polish Academy of Science, Golysz, Poland. Prior to the experiments, fish were adapted for 4 weeks at 21 °C and 12L:12D light/dark cycle in recirculating tap water at the Institute of Zoology and Biomedical Research in Krakow, Poland. Fish were kept in tanks (volume 375 l, flow rate 4 l/min, density 45 fish/tank and 60 g/l), and all unnecessary interferences were avoided. Fish were fed pelleted dry food (Aller Master, Aller Aqua, Poland) at a daily maintenance rate of 1% of their estimated body weight. In order to avoid additional stress and/or differences in handling, all samplings were performed by the same person and at the same time of day (at 9.00 a.m.). All animals were handled in strict accordance with good animal practice as defined by the relevant national and local animal welfare bodies, and procedures were approved by the local ethical committee (2nd Local Institutional Animal Care and Use Committee (IACUC) in Krakow, Poland, license number 292/2017).

### 2.2. In vitro study

#### 2.2.1. Cell isolation

Fish were bled through puncture of the caudal vein. Head kidney and trunk kidney cell suspensions were obtained by passing through 100 μm nylon mesh (BD Biosciences USA) with cRPMI (cRPMI 1640, Invitrogen, Carlsbad, CA), adjusted to carp osmolarity of 270 mOsm/$kg^{-1}$ with distilled water) containing 0,051 mg/ml heparin (Leo Pharmaceutical Products Ltd., Weesp, the Netherlands) and washed once for 10 min at 800 x g. This cell suspension was layered on a discontinuous Percoll (GE Healthcare, Sweden) gradient (1.020, 1.060, 1.070 and 1.083 g/$cm^3$) [18] and centrifuged for 30 min at 800 x g with the brake disengaged. The layer 1.060 g/$cm^3$ (monocytes/macrophages enriched cell population) was collected.

To verify the purity of the isolated cell populations, monocytes/macrophages enriched cell population was stained with neutral red [NR, 0.1 mg/ml, 3 min, at RT (Sigma-Aldrich, USA)] or with Türk's solution [0.01% crystal violet (Sigma-Aldrich, USA) in 3% acetic acid (Chempur, Poland), 3 min, at RT] and analyzed in hemocytometer under the light microscope (400× magnification, Meiji Techno, USA). Pictures were taken Moticam10 10.0 MP (China). Images were analyzed in Gimp 2.10 (Fig. 1S). Additionally, with a FACScalibur flow cytometer (BD Biosciences) 30000 threshold events per sample were analyzed for their forward scatter (FSC) (for cell size) and sideward scatter (SSC) (cell complexity) profiles. Data were analyzed using WinMDI 2.9 software (Joe Trotter, http://facs.scripps.edu).

As described previously [18] we also found that in control fish, cell fraction obtained from the head kidney contained an abundance of macrophages (48.86% ± 1.38) and that it did not differ significantly from the cell fractions obtained from the trunk kidney (49.42% ± 3.46). Similar percentages of monocytes/macrophages were also obtained from both organs of stressed fish (44.57% ± 1.46 and 51.73% ± 1.38, respectively).

Moreover, in isolated cell fractions enriched in: lymphocytes (1.020 g/$cm^3$), monocytes/macrophages (1.060 g/$cm^3$) and neutrophilic granulocytes (1.070 g/$cm^3$) gene expression of markers for neutrophilic granulocytes (*mpx*), T-lymphocytes (*lck*) and B-lymphocytes (*igm heavy chain*) were measured by RQ-PCR as described in chapter 2.4. Although gene expression of *mpx, lck* and *igm heavy chain* was found in the monocyte/macrophage fraction, it was considerably

lower than the expression measured in neutrophils (mpx) and in lymphocytes (lck, IgM heavy chain) and did not differ between monocyte/macrophage enriched fraction from the head and trunk kidney (Fig. 1S).

#### 2.2.2. Cell culture and in vitro stimulation

The monocyte/macrophage enriched suspension was resuspended in carp cRPMI + + (cRPMI supplemented with 1.5% (v/v) pooled carp serum with antibiotics (1% L-glutamine (Sigma Aldrich, St Louis, MO), 1% (v/v) penicillin G (Sigma Aldrich, St Louis, MO) and 1% (v/v) streptomycin sulphate (Sigma Aldrich, St Louis, MO)) to a density of 10 million cells per ml. Monocytes/macrophages were seeded in 24-well cell culture plates (Nest Biotech Co, China) at 27 °C, 5% $CO_2$, and stimulated for 6 h with lipopolysaccharide (LPS, Escherichia coli serotype O55: B5, Sigma–Aldrich, St. Louis, MO, L2880, 30 μg/ml), cortisol (CORT, Sigma–Aldrich, St. Louis, MO, 1 μM), their combination (CORT + LPS), N-terminal ANXA1-derived peptide annexin 1-(2–26) (Ac2-26, 10 μg/ml, Tocris Bioscience, USA) or its combination with LPS (LPS + Ac2-26). Control cells (C) were treated with the same volume of culture medium or 1% DMSO (Bioshop, Canada) as used for experiments with cortisol and Ac2-26, respectively. After 6 h of stimulation cells were suspended in 350 μl RL buffer (Eurex, Gdansk, Poland) with 1% β2-mercaptethanol (Sigma–Aldrich, St. Louis, MO) and kept in −80 °C for further analyses.

### 2.3. Stress model

Prolonged restraint (24 h) was given by netting the fish and suspending the nets with the fish in separate tanks with a volume of 50 l [19]. After 24 h, the fish of the experimental group were immediately (within 30 s) transferred all at once to a tank with 0.2 g/l tricaine methane sulfonate (TMS, Sigma-Aldrich, St. Louis, MO, USA) buffered with 0.4 g/1 $NaHCO_3$ (POCH, Gliwice, Poland) resulting in rapid (< 1 min) and deep anesthesia. A control group was housed in an identical tank but left undisturbed. Control fish were sampled following rapid netting and anesthesia, immediately before sampling of the experimental group.

Fish were bled and monocytes/macrophages from the head- and trunk kidney of control and stressed fish were isolated as described earlier (chapter 2.2.1.).

Monocyte/macrophage suspensions were divided. One half of the cells was immediately resuspended in RL buffer with 1% β2-mercaptethanol and kept at −80 °C for further analyses. The remaining half of the cell suspension was used for *ex vivo* experiments as described in chapter 2.3.3.

#### 2.3.1. Cortisol assay

Blood was collected in covered test tubes and allowed to clot overnight at 4 °C. Blood clots were removed by centrifuging at 3000 x g for 30 min and the serum was collected and stored at −20 °C for future use. The free cortisol levels were determined using DetectX®, a cortisol enzyme immunoassay kit (with a detection limit at 45.4 pg/ml), according to the manufacturer's protocol (Arbor Assays, Ann Arbor, Michigan, USA). All standards and samples from every individual fish were analyzed in duplicate, in the same batch.

#### 2.3.2. Glucose determination

The level of glucose in blood serum was measured as described by Dubowski [20]. Briefly, 6% ortho-toluidine (Sigma-Aldrich Co., St. Louis, MO, USA) in glacial acetic acid (POCH SA, Gliwice, Poland) was used as the reagent for glucose, after deproteinization with 3% trichloroacetic acid (Sigma-Aldrich Co., St. Louis, MO, USA). The serum was mixed with the reagent in a ratio of 1:3. The reaction mixture was heated at 100 °C for 10 min, rapidly cooled to room temperature and measured spectrophotometrically. The absorbance was determined at 630 nm with an ELISA microplate reader (ASYS Hitech GmbH Expert Plus). Glucose (POCH, Gliwice, Poland) standards containing 0.03–1 mg/ml in deionized $H_2O$ were prepared.

#### 2.3.3. Ex vivo study

Monocytes/macrophages from control and stressed fish were resuspended to a density of 10 million cells per ml in carp cRPMI + + and seeded in 24-well cell culture plates. Cells were *ex vivo* stimulated for 6 h with LPS (30 μg/ml) and after stimulation suspended in 350 μl RL buffer with 1% β2-mercaptethanol and kept at −80 °C for further analyses.

### 2.4. Expression of genes by RQ-PCR

#### 2.4.1. RNA isolation

RNA from HK and TK monocytes/macrophages was isolated using the GeneMATRIX Universal RNA Purification Kit (Eurex, Gdansk, Poland) according to the manufacturer's protocol. Final elution was carried out in 30 μl of nuclease-free water, to maximize the concentration of RNA. RNA concentrations were measured by Tecan Spark NanoQuant Plate™. Samples were stored at −80 °C.

#### 2.4.2. cDNA synthesis

For each sample, a non-RT (non-reverse transcriptase) control was included. The cDNA synthesis reaction was performed using High-Capasity cDNA Reverse Transcription Kits (Applied Biosystems, USA) using the manufacturers protocol. Briefly, 1 μg of total RNA was added to 10 μl RT master mix containing 2 μl 10X RT Buffer, 0.8 μl 25XdNTP Mix (100 mM); 2 μl 10XRT Random Primers; 1 μl MultiSribe™Reverse Transcriptase and 4.2 μl of nuclease-free water. Samples were then placed into the thermal cycler (Ditabis AG, Pforzheim, Germany, 25 °C at 10 min; 37 °C at 120 min; 85 °C at 5 min followed by rapid cooling to 4 °C).

Samples were set at 100 μl with dematerialized water and stored at −20 °C until further use.

#### 2.4.3. Real-time quantitative PCR

The following carp-specific primers (5′–3′) for putative markers of M1 macrophage polarization were used: iNOS, IL-1β, IL-12p35, CXCL8_L1, CXCL8_L2, CXCb1, CXCb2, CXC receptors: CXCR1-3. As putative M2 polarization markers primers for arginase 1 and 2, IL-10, MMP-9 as well as glucocorticoid receptors (GR1 and 2) were used. The 40S ribosomal protein s11 gene served as an internal standard (accession numbers and primer sequences are listed in Table 1S).

For RQ-PCR 4 μl cDNA and forward and reverse primers (2 μl each) were added to 7 μl SYBR®Select Master Mix (Applied Biosystems, USA). RQ-PCR (2 min at 50 °C, 2 min at 95 °C, 40 cycles of 15 s at 95 °C, 60 s at 60 °C) was carried out with a Rotor-Gene Q (Qiagen, Hilden, Germany). Following each run, melt curves were collected by detecting fluorescence from 60 to 90 °C at 1 °C intervals.

Constitutive expression was rendered as a ratio of target gene vs. reference gene (40S ribosomal protein s11 gene) and was calculated according to the following equation:

$$\text{Ratio} = \frac{(E_{reference})^{Ct_{reference}}}{(E_{target})^{Ct_{target}}}$$

where E is the amplification efficiency and Ct is the number of PCR cycles needed for the signal to exceed a predetermined threshold value [21].

Changes in the gene expression upon stress and/or cell stimulation were rendered as a ratio of target gene vs. reference gene (40S ribosomal protein s11 gene) relative to expression in control samples according to the following equation:

$$\text{Ratio} = \frac{(E_{target})^{\Delta Ct}Target^{(control-sample)}}{(E_{reference})^{\Delta Ct}Reference^{(control-sample)}}$$

### 2.5. Cell activity

Head and trunk kidney monocytes/macrophages from control and stressed fish were treated as described in 2.2.2 and 2.3.3. and cultured for 24 h.

#### 2.5.1. Nitric oxide release

Nitrite/nitrate production, an indicator of nitric oxide synthesis, was measured in cell culture supernatants as described previously [22]. Briefly, 50 µl cell culture supernatant was added to 25 µl 1% (w/v) sulphanilamide in 2.5% (v/v) phosphoric acid and 25 µl of 0.1 (w/v) N-naphthyl-ethylenediamine in 2.5% (v/v) phosphoric acid (all from Sigma-Aldrich, St. Louis, MO). The OD reading at 540 nm (with 690 nm as a reference) was taken using cRPMI$^{++}$ medium as blank. Nitrite concentration was calculated by comparison with a sodium nitrite standard curve.

#### 2.5.2. Arginase activity

Arginase activity was measured as described by Corraliza et al. (1994) [23]. Cells were lysed in 50 µl of 0.1% Triton X-100 containing 5 µg of pepstatin, 5 µg of aprotinin, and 5 µg of antipain at room temperature for 30 min. 35 µl of 10 mM $MnCl_2$ and 50 mM Tris-HCl (pH 7.5) was added, and the mixture was incubated for 20 min at 55 °C. To 50 µl of this activated lysate, 50 µl of 0.5 M L-arginine (pH 9.7) was added and incubated for 1 h at 37 °C. The reaction was stopped by adding 400 µl of an acid mixture containing $H_2SO_4$, $H_3PO_4$, and $H_2O$ (1:3:7), then to each reaction 25 µl of 9% α-isonitrosopropiophenone (in 100% ethanol) was added and incubated for 45 min at 100 °C. After 10 min cooling in the dark, the OD was read at 540 nm, and the arginase activity was calculated by comparison with a urea standard curve.

### 2.6. Statistical analysis

Data were expressed as mean and standard error (SE). Differences in the serum level of cortisol and glucose in control and stressed animals as well as differences in the constitutive gene expression between HK and TK-derived cells were compared using the Mann-Whitney *U* test. The changes in gene/expression/cell activity were compared by a two-way analysis of variance (ANOVA), followed by post hoc Tukey's test applied for the same data. The differences were considered statistically significant at $p < 0.05$.

## 3. Results

### 3.1. Stress induces changes in the level of cortisol and glucose

As expected, restraining stress induced a significant increase in blood serum cortisol levels (from 15.84 ± 0,57 ng/ml in control animals to 40.12 ± 8.96 ng/ml in stressed fish) and in the level of glucose (from 142.92 ± 18.14 mg/dl in control animals to 332.68 ± 41.35 mg/dl in stressed fish).

### 3.2. Effects of cortisol in vitro on the expression of pro-inflammatory mediators

Monocytes/macrophages derived from the head kidney showed lower constitutive expression of *il-1β* gene than cells derived from the trunk kidney (Fig. 1A).

Moreover, when stimulated with LPS, both HK and TK monocytes/macrophages up-regulated gene expression of *inos* (Fig. 1B), *il-1β* (Fig. 1C) and *il-12p35* (Fig. 1D). However, the LPS-stimulated up-regulation of *inos* was significantly higher in TK-derived cells than in cells

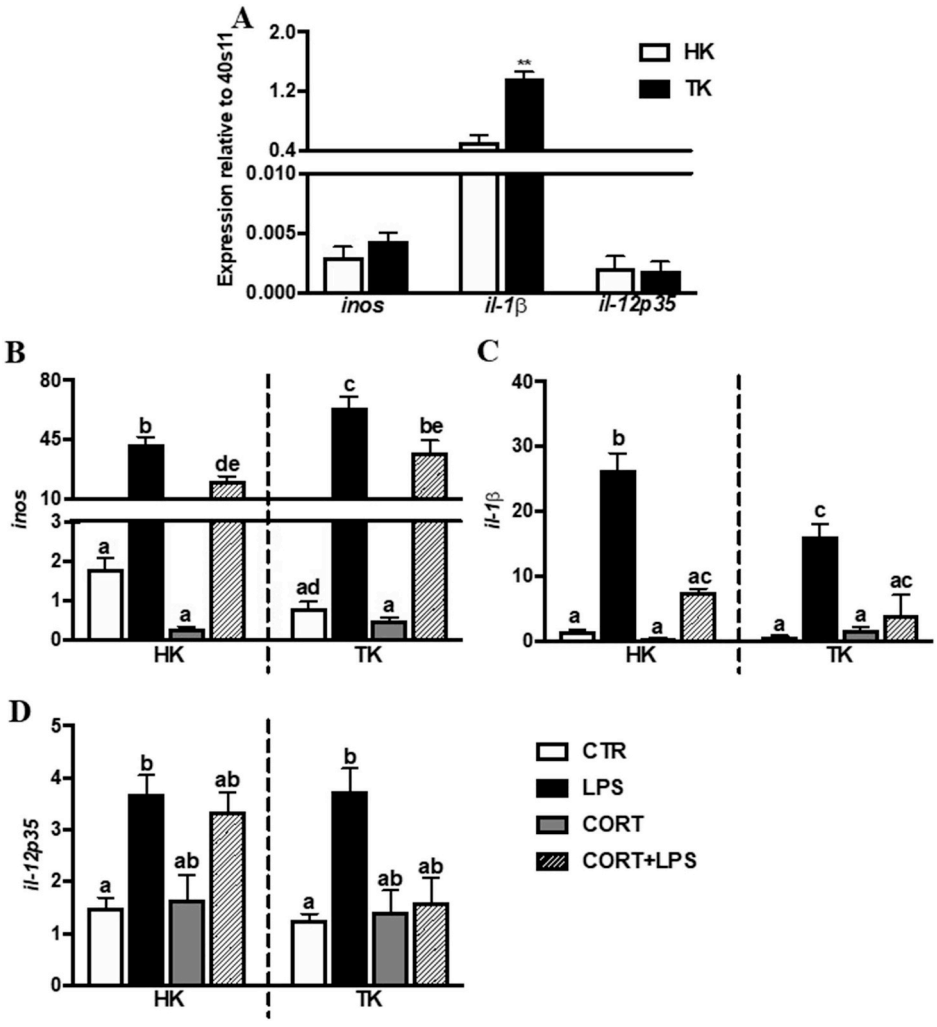


**Fig. 1.** Gene expression of pro-inflammatory mediators in the head kidney (HK) and trunk kidney (TK) monocytes/macrophages. A) constitutive gene expression, B-D) changes in gene expression in cells treated *in vitro* for 6 h with lipopolysaccharide (LPS, 30 µg/ml), cortisol (CORT, 1 µM) or their combination (CORT + LPS). Constitutive gene expression was determined by quantitative RT-PCR and expressed relative to the expression of the 40S ribosomal protein s11 gene. Changes in gene expression are shown as x-fold increase compared to un-stimulated cells (RPMI-treated) and standardized for the housekeeping gene 40S ribosomal protein s11. Averages and S.E (n = 4–5). Stars indicate significant differences between HK and TK-derived cells (**p ≤ 0.01). Mean values not sharing letters are statistically different according ANOVA.

from the HK (Fig. 1B), while the LPS-induced up-regulation of *il-β* was lower in TK cells than in cells derived from HK (Fig. 1C). Compared to control untreated cells, no significant changes in the expression of *inos, il-1β* and *il-12p35* were observed in HK and TK monocytes/macrophages treated with cortisol only (Fig. 1B–D), while in cells treated with LPS and cortisol significant down-regulation of the expression of *inos* (Fig. 1B) and *il-1β* (Fig. 1C) was observed compared to cells treated with LPS only.

### 3.3. Effects of cortisol in vitro on the expression of CXC chemokines and their receptors

Monocytes/macrophages derived from the head and trunk kidney did not differ in their constitutive expression of genes encoding CXCL8 and CXCb chemokines (Fig. 2A). In both HK and TK monocytes/macrophages, LPS up-regulated the gene expression of *cxcl8_l2* and *cxcb2* chemokines (Fig. 2C and E), but not the expression of the gene encoding CXCb1 (Fig. 2D). LPS-induced upregulation was also observed for *cxcl8_l1* gene expression in HK cells (Fig. 2B). Interestingly, LPS-induced up-regulation of *cxcl8_l2* was higher in the TK than in the HK cells (Fig. 2C). Gene expression of the CXCL8 and CXCb chemokines did not differ between control untreated cells and cells treated with cortisol only (Fig. 2B–E). However, in HK monocytes/macrophages treated with LPS + cortisol, a significant down-regulation of the expression of *cxcl8_l1* (Fig. 2B), *cxcl8_l2* (Fig. 2C) and *cxcb2* (Fig. 2E) was observed compared to cells treated with LPS only. In TK monocytes/macrophages treated with LPS + cortisol, down-regulation of the expression of *cxcl8_l2* (Fig. 2C) was found.

Monocytes/macrophages derived from the head kidney did not differ in their constitutive expression of genes encoding for *cxcr1* and *cxcr2,* while the expression of *cxcr3* was lower in the head kidney-derived cells than in cells derived from the trunk kidney (Fig. 3A).

LPS did not change the gene expression of *cxcr1-3* in HK monocytes/macrophages, while it down-regulated the expression of *cxcr3* in TK cells (Fig. 3B–D). Cortisol alone or in combination with LPS did not affect the expression of *cxcr1-3* (Fig. 3B–D).

### 3.4. Effects of cortisol in vitro on the expression of anti-inflammatory mediators and GR receptors

Monocytes/macrophages derived from the head kidney showed lower constitutive expression of genes encoding *arginase 1* and *arginase 2,* than cells derived from the trunk kidney (Fig. 4A). Cells from both sources did not differ in their constitutive expression of genes encoding *il-10*, *mmp-9* (Fig. 4A) as well as *gr1* and *gr2* (Fig. 5A).

Moreover, in HK monocytes/macrophages, LPS up-regulated the gene expression of *arginase 2* (Fig. 4C), while both in HK and TK cells this LPS stimulation up-regulated the expression of *il-10* (Fig. 4D).

In HK monocytes/macrophages, cortisol did not change the gene expression of *arginase 1, arginase 2*, *il-10*, *mmp-9* and both *gr* receptors (Figs. 4 and 5), while it up-regulated *arginase 1* (Fig. 4B) and *mmp-9* (Fig. 4D) gene expression TK cells. In HK monocytes/macrophages treated with LPS + cortisol a significant down-regulation of the expression of *arginase 2* (Fig. 4C) was observed compared to cells treated

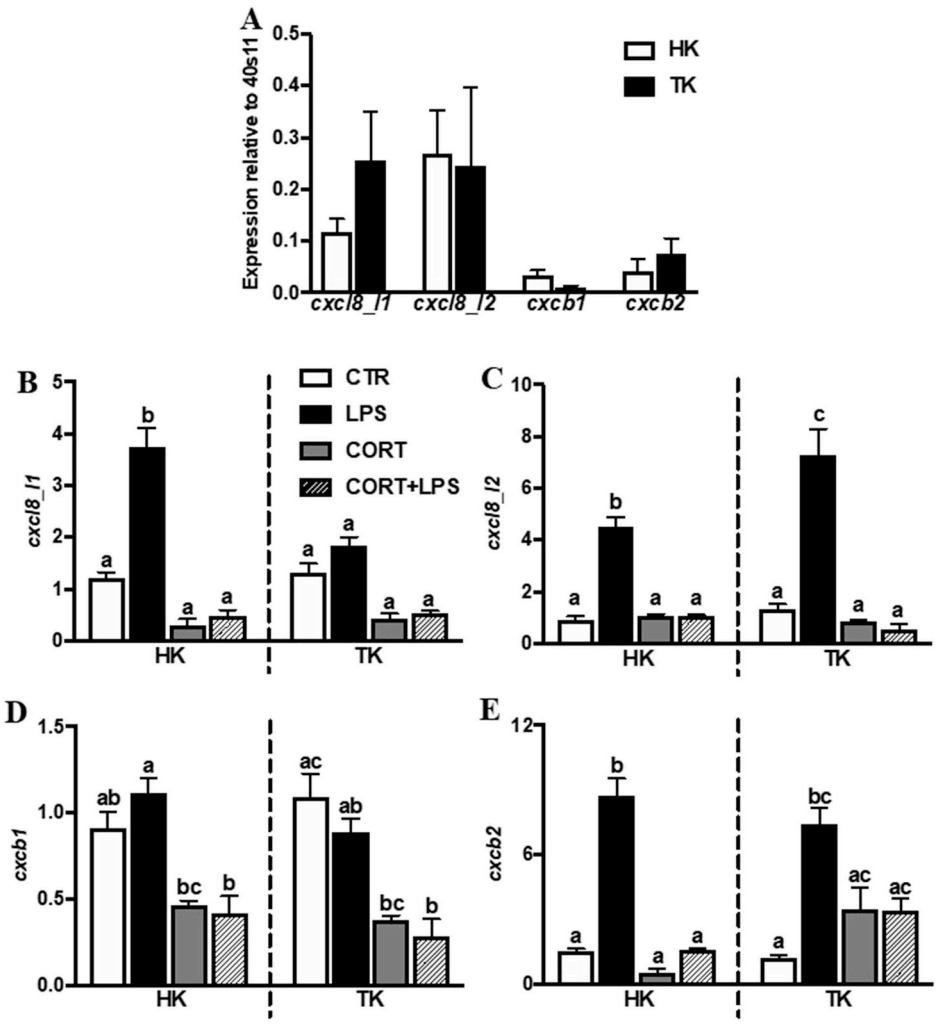


**Fig. 2.** Gene expression of CXC chemokines in the head kidney (HK) and trunk kidney (TK) monocytes/macrophages. A) constitutive gene expression, B-E) changes in the gene expression in cells treated *in vitro* for 6 h with lipopolysaccharide (LPS, 30 μg/ml), cortisol (CORT, 1 μM) or their combination (CORT + LPS). Constitutive gene expression was determined by quantitative RT-PCR and expressed relative to expression of the 40S ribosomal protein s11 gene. Changes in gene expression are shown as x-fold increase compared to un-stimulated cells (RPMI-treated) and standardized for the housekeeping gene 40S ribosomal protein s11. Averages and S.E (n = 4–5). Mean values not sharing letters are statistically different according ANOVA.

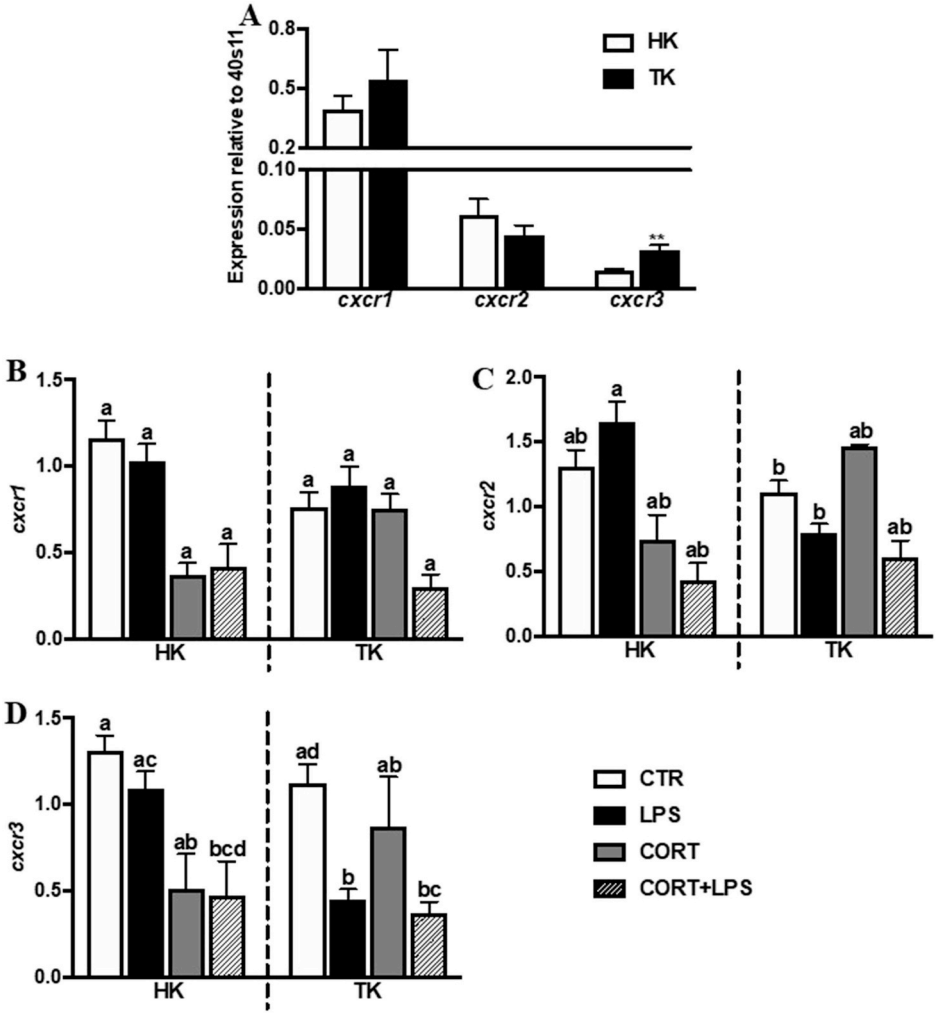


**Fig. 3.** Gene expression of CXC chemokine receptors in the head kidney (HK) and trunk kidney (TK) monocytes/macrophages. A) constitutive gene expression, B-D) changes in the gene expression in cells treated *in vitro* for 6 h with lipopolysaccharide (LPS, 30 μg/ml), cortisol (CORT, 1 μM) or their combination (CORT + LPS). Constitutive gene expression was determined by quantitative RT-PCR and expressed relatively to expression of the 40S ribosomal protein s11 gene. Changes in the gene expression are shown as x-fold increase compared to un-stimulated cells (RPMI-treated) and standardized for the housekeeping gene 40S ribosomal protein s11. Averages and S.E (n = 4–5). Stars indicate significant differences between HK and TK-derived cells (**p ≤ 0.01). Mean values not sharing letters are statistically different according ANOVA.

with LPS only. In TK monocytes/macrophages treated with LPS + cortisol down-regulation of the expression of *il-10* (Fig. 4D) and up-regulation of *arginase 1* (Fig. 4B) and both *gr1* and *gr2* (Fig. 5B and C) was found.

### 3.5. Effects of cortisol in vitro on monocyte/macrophage activity

Monocytes/macrophages from HK and TK did not differ in their level of NO release, while the activity of arginase was significantly higher in TK than in HK cells. As expected, LPS increased the synthesis/release of NO from both HK and TK monocytes/macrophages, while in HK cells treated with LPS + cortisol, a decreased NO level was observed compared to cells treated with LPS alone (Fig. 6A). Neither LPS nor cortisol alone induced changes the arginase activity of HK and TK monocytes/macrophages, while in LPS + cortisol-treated TK cells a lower arginase activity was found than in cells treated with LPS only (Fig. 6B).

### 3.6. Effects of Ac2-26 in vitro on the expression of pro- and anti-inflammatory mediators

In Ac2-26-treated HK and TK monocytes/macrophages, gene expression of pro- (*inos, il-1β, il-12p35*) and anti-inflammatory (*arginase 2, il-10*) mediators was measured. No significant changes in the gene expression of these mediators were found in cells treated with Ac2-26 compare to control DMSO-treated cells. Moreover, Ac2-26 did not change the expression of these genes in LPS-treated HK monocytes/macrophages, while in TK cells it only up-regulated the expression of *arginase 2* gene (data not shown).

### 3.7. Effects of restraint stress in vivo on the gene expression of pro- and anti-inflammatory mediators and chemokines

Freshly isolated HK monocytes/macrophages from stressed animals showed higher gene expression of *arginase 1* (Fig. 7A), *il-10* (Fig. 7C) and *mmp-9* (Fig. 7D) than HK cells derived from control, unstressed fish. At the same time, cells derived from the trunk kidney of stressed fish showed higher expression of *cxcr3* (Fig. 2S) and *mmp-9* (Fig. 7D). No significant stress-induced changes were observed in the gene expression of pro-inflammatory cytokines and CXC chemokines and GRs (Fig. 2S).

### 3.8. Effects of restraint stress on the ex vivo LPS sensitivity of monocytes/macrophages

In LPS-stimulated HK and TK cells from stressed animals, the expression of *cxcl8_l1* was higher than in stimulated cells from control fish. Moreover, in LPS-treated TK cells from stressed animals higher expression of *cxcl8_l2, cxcb2* and gr2 but lower expression of *il-12p35* and *arginase 2* was found compare to LPS-treated cells derived from unstressed fish (Table 1).

Moreover, both in HK and TK monocytes/macrophages from control and stressed fish that were treated *ex vivo* with LPS, increased NO levels were observed (Fig. 3S). Moreover, TK-derived monocytes/macrophages from control fish showed higher arginase activity than their

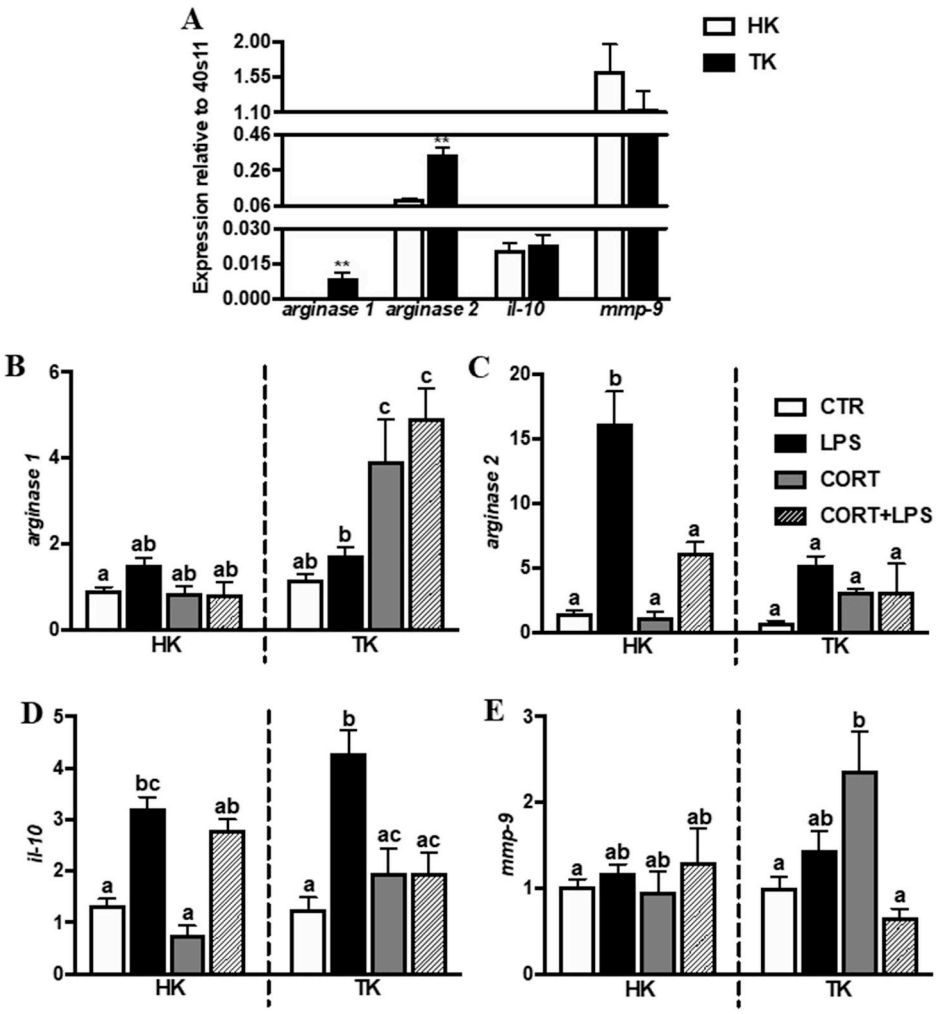


**Fig. 4.** Gene expression of anti-inflammatory mediators in the head kidney (HK) and trunk kidney (TK) monocytes/macrophages. A) constitutive gene expression, B-E) changes in the gene expression in cells treated *in vitro* for 6 h with lipopolysaccharide (LPS, 30 μg/ml), cortisol (CORT, 1 μM) or their combination (CORT + LPS). Constitutive gene expression was determined by quantitative RT-PCR and expressed relatively to expression of the 40S ribosomal protein s11 gene. Changes in the gene expression are shown as x-fold increase compared to un-stimulated cells (RPMI-treated) and standardized for the housekeeping gene 40S ribosomal protein s11. Averages and S.E (n = 4–5). Stars indicate significant differences between HK and TK-derived cells (**$p \leq 0.01$). Mean values not sharing letters are statistically different according ANOVA.

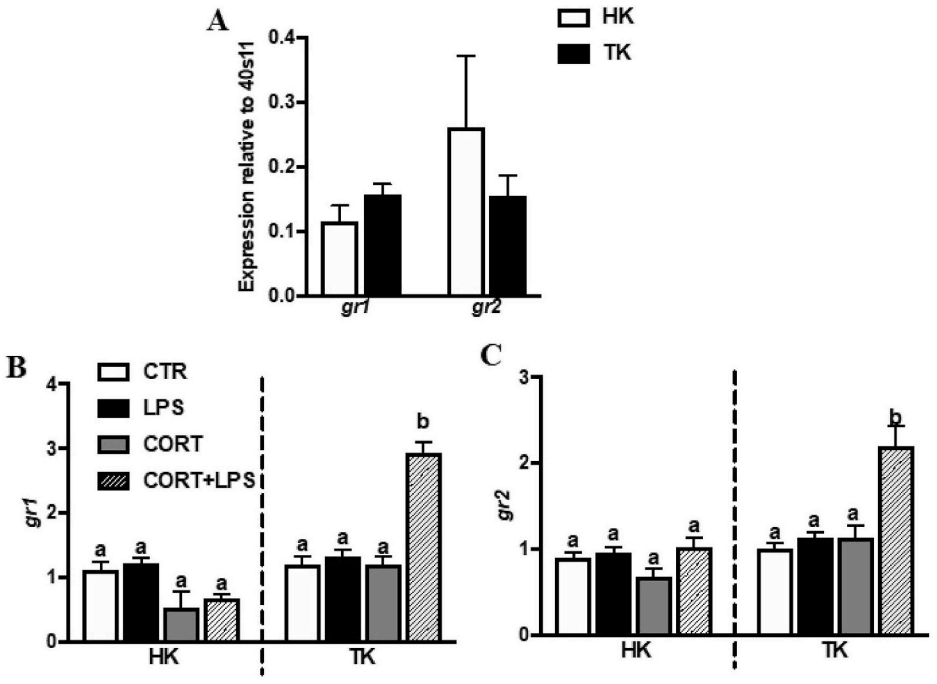


**Fig. 5.** Gene expression of glucocorticoid receptors in the head kidney (HK) and trunk kidney (TK) monocytes/macrophages. A) constitutive gene expression, B–C) changes in the gene expression in cells treated *in vitro* for 6 h with lipopolysaccharide (LPS, 30 μg/ml), cortisol (CORT, 1 μM) or their combination (CORT + LPS). Constitutive gene expression was determined by quantitative RT-PCR and expressed relative to expression of the 40S ribosomal protein s11 gene. Changes in the gene expression are shown as x-fold increase compared to un-stimulated cells (RPMI-treated) and standardized for the housekeeping gene 40S ribosomal protein s11. Averages and S.E (n = 4–5). Mean values not sharing letters are statistically different according ANOVA.

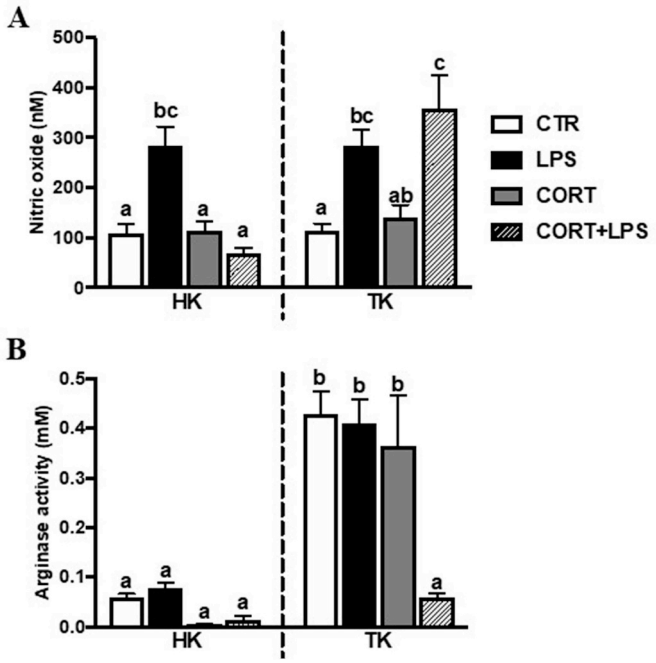


**Fig. 6.** *In vitro* effects of cortisol (1 μM, CORT) on nitrite release (A) and arginase activity (B) in control unstimulated (CTR) head kidney (HK) and trunk kidney (TK) monocytes/macrophages and/or cells stimulated (24 h) with lipopolysacharide (30 μg/ml, LPS). Averages and S.E (n = 4–5). Mean values not sharing letters are statistically different according ANOVA.

counterparts from HK. Both in HK and TK cells, no changes in arginase activity upon *ex vivo* LPS-treatment were observed. In contrast, TK cells from stressed animals showed lower arginase activity than cells from control unstressed fish (Fig. 3S).

**Table 1**
Stress-induced changes in the gene expression of pro-inflammatory mediators, CXC chemokines and their receptors, anti-inflammatory mediators and glucocorticoid receptors in the head kidney (HK) and trunk kidney (TK) monocytes/macrophages treated *ex vivo* for 6 h with lipopolysaccharide (LPS, 30 μg/ml). Changes in gene expression are shown as x-fold increase compared to gene expression measured in LPS-stimulated monocytes/macrophages from unstressed fish (solid line at 1) and standardized for the housekeeping gene 40S ribosomal protein s11. Averages and S.E (n = 4–5). Stars indicate significant differences between LPS-treated cells derived from unstressed and stressed fish (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001). Number signs indicate significant differences between HK and TK cells (#p ≤ 0.05, ##p ≤ 0.01, ###p ≤ 0.001).

| gene | HK | TK |
|---|---|---|
| *inos* | 1.04 ± 0.08 | 1.27 ± 0.38 |
| *il-1β* | 1.46 ± 0.34 | 1.30 ± 0.41 |
| *il-12p35* | 0.43 ± 0.13 | 0.36 ± 0.10 ** |
| *cxcl8_l1* | 2.20 ± 0.38 ** | 2.12 ± 0,33 * |
| *cxcl8_l2* | 0.66 ± 0.13 | 3.78 ± 0.65 *, # |
| *cxcb1* | 0.84 ± 0.12 | 0.65 ± 0.16 |
| *cxcb2* | 1.16 ± 0.18 | 2.00 ± 0.23 * |
| *cxcr1* | 0.69 ± 0.09 | 1.33 ± 0.21 # |
| *cxcr2* | 0.89 ± 0.14 | 1.44 ± 0.12 |
| *cxcr3* | 0.72 ± 0.22 | 1.56 ± 0.31 |
| *arginase 1* | 1.09 ± 0.19 | 0.26 ± 0.06 |
| *arginase 2* | 1.41 ± 0.20 | 0.19 ± 0,01 *, ### |
| *il-10* | 1.41 ± 0.23 | 1.11 ± 0.17 |
| *mmp-9* | 0.48 ± 0.12 | 1.43 ± 0.37 |
| *gr1* | 0.98 ± 0.11 | 0.80 ± 0.07 |
| *gr2* | 1.48 ± 0.06 | 2.43 ± 0.43 *, # |

## 4. Discussion

We focused on the effects of cortisol *in vitro* and of prolonged restraint stress *in vivo,* on the polarization of monocytes/macrophages from two hematopoietic organs: the head- and trunk kidney. Although the trunk kidney monocytes/macrophages show higher constitutive

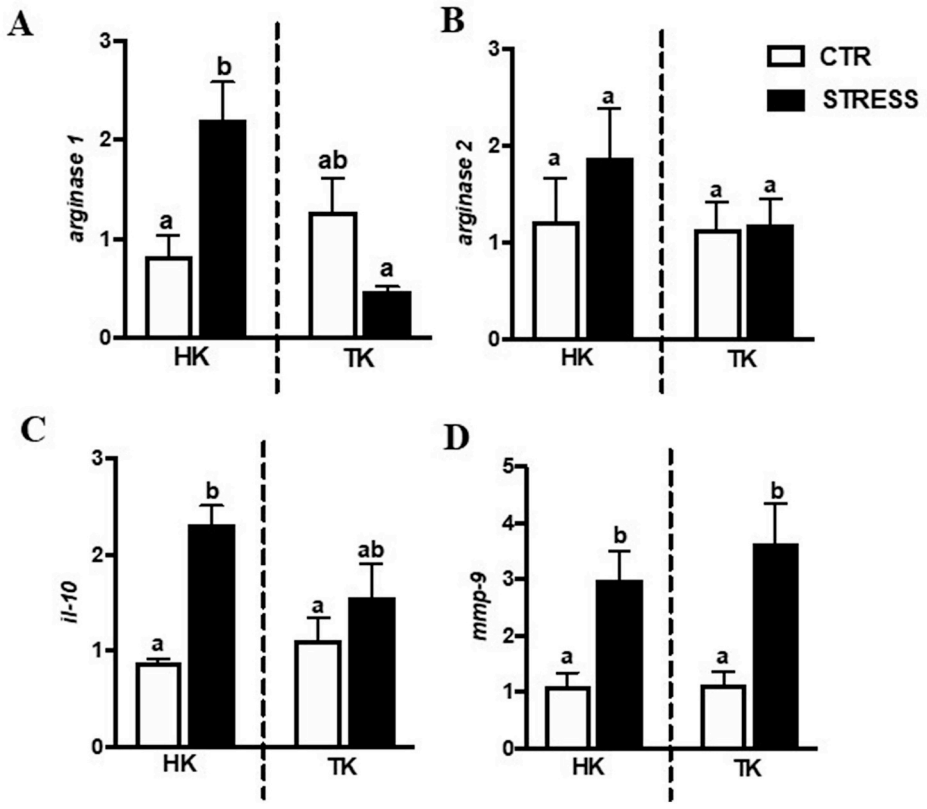


**Fig. 7.** Stress-induced changes in the gene expression of anti-inflammatory mediators in the head kidney (HK) and trunk kidney (TK) monocytes/macrophages. Changes in the gene expression are shown as x-fold increase compared to gene expression measured in monocytes/macrophages from unstressed fish (CTR) and standardized for the housekeeping gene 40S ribosomal protein s11. Averages and S.E (n = 4–5). Mean values not sharing letters are statistically different according ANOVA.

expression of genes involved in macrophage polarization (*il-1b* and *cxcr3* as well as both arginase genes) as well as higher arginase activity, the general pattern of their *in vitro* response to LPS was similar: up-regulation of the expression of the majority of crucial genes, except for the chemokine CXC receptors and increased NO release. Also, Joerink et al. [24] compared the activity of monocytes/macrophages from both head and trunk kidney of carp. Similar to our observations, they found that freshly isolated trunk kidney leukocytes showed higher arginase activity than head kidney leukocytes, while the production of NO was comparable in cells from both sources. Moreover, in head kidney-derived macrophages of carp that were cultured for 6 days, stimulation with LPS induced an up-regulation of the gene expression of M1 markers (TNF-α, IL-1β, IL-12p35, iNOS, CXCL8_L1 (CXCa)) [11]. Interestingly, LPS up-regulated the gene expression of both pro- and anti-inflammatory markers. Similar effects of LPS on the level of IL-10 were previously observed both in human primary macrophages and in the mouse macrophage cell line RAW264.7 [25,26]. Most probably, the Sp1 (specificity protein 1) transcription factor is a central mediator of LPS-induced IL-10 up-regulation [25–28]. Also, LPS-stimulated increase of arginase expression and activity was observed previously. For example, Wang et al. [29] found that in LPS-stimulated RAW 264.7 macrophages, the activity of arginase increased linearly. Furthermore, the study of Jin et al. [30] showed that LPS stimulates both the gene expression and the protein level of iNOS, arginase 1 and arginase 2. Moreover, they revealed that both ERK and p38 are involved in LPS-stimulated arginase 2 up-regulation, while iNOS induction is dependent on the activation of p38 only. Also, in teleost fish leukocytes, LPS-induced changes in the p38 MAPK pathway [31,32] and IL-10 expression were detected [33–35]. In our hands, LPS-induced the up-regulation of *arginase 2* and *il-10* gene expression [36] in the head kidney phagocytes.

Furthermore, we found that the cells from both sources did not differ in their constitutive expression of the *gr1* and *gr2* genes and in *in vitro* response to LPS, whereas they significantly differ in the response to cortisol and stress. In LPS-stimulated monocytes/macrophages from HK, cortisol down-regulated the expression of almost all pro-inflammatory cytokines/chemokines - markers of classical (M1) macrophage polarization and decreased production of NO, while in TK cells it only down-regulated the expression of *inos* and *cxcl8_l2*.

It is therefore concluded that in LPS-stimulated head kidney-derived macrophages, cortisol *in vitro* counteracts the action of the stimulant.

Previously, cortisol mediated antagonism of LPS-induced inflammatory gene expression has been reported. For example, in LPS-treated murine RAW264.7 macrophages, cortisol significantly suppressed the production of prostaglandin E2 (PGE2) and decreased the gene and protein expression of iNOS and cyclooxygenase-2 (COX-2). Moreover, it inhibited the mRNA expression of pro-inflammatory cytokines, including TNFα, IL-1β, and IL-6 and it decreased IL-1β secretion [37]. Similarly, carp head kidney phagocytes treated *in vitro* with cortisol inhibited NO production and down-regulated gene expression of *il-1 β, tnfα, inos* and *il-12p35* and serum amyloid A [38,39]. Furthermore, in *Vibrio anguillarum* bacterin-stimulated gilthead sea bream head kidney primary cells, cortisol down-regulated gene expression of *il-1β, il-6, tnfα* and *il-10*. The GR blocker – mifepristone restored this effect to basal expression levels for *il-1β* and *tnf-α* only [40]. Cortisol also inhibited the LPS-dependent induction of the expression of pro-inflammatory cytokines (TNF-α2, IL-1β) in head kidney macrophages of trout that were cultured for 5 days [32]. A microarray analysis by these authors revealed that a simultaneous cortisol and LPS stimulation led to an overall antagonism of the actions of LPS on the macrophage transcriptome (more than 80% of LPS-regulated genes).

Especially interesting differences between the HK- and TK-derived monocytes/macrophages were observed for *arginase 1* expression. Whereas in HK cells treated with LPS + cortisol no changes of *arginase 1* gene expression were observed, TK cells that were treated similarly, showed up-regulation of *the arginase* 1 mRNA. This up-regulation correlated with the up-regulation of *gr1* and *gr2* gene expression.

Surprisingly, Ac2-26 did not affect the expression of M1 and M2 markers in carp macrophages. Previous *in vitro* studies showed that both Ac2-26 as well glucocorticoid-regulated protein - annexin A1, blocked the expression of *iNOS* in J774.2 mouse cells stimulated with lipopolysaccharide [41]. Moreover, in LPS-pretreated J774.2 macrophages, annexin A1 stimulated the production of IL-10 [16].

Furthermore, we studied how prolonged restraint stress affects the expression of M1 and M2 markers in head- and trunk-kidney macrophages. Interestingly, in freshly isolated head kidney cells, up-regulation of the gene expression of M2 markers *(arginase 1, il-10* and *mmp-9*) was observed. At the same time, freshly isolated trunk kidney macrophages from stressed animals showed only up-regulation of the *cxcr3* and *mmp-9* genes.

LPS-stimulated HK macrophages from stressed animals showed only minor differences in the gene expression of M1 and M2 markers compared to LPS-treated monocytes/macrophages from control fish, while stress-induced changes in TK-derived LPS-treated cells were more pronounced. However, these changes did not clearly indicate whether in TK monocytes/macrophages stress will stimulate classical or alternative polarization as gene expression of pro-inflammatory chemokines was up-regulated, but both the pro-inflammatory *il-12p35* gene and anti-inflammatory *arginase 2* were down-regulated.

Previous studies in fish indicated that stress/glucocorticoids *in vivo* affect the activity of macrophages. For example, in catfish, intraperitoneal injection with LPS and dexamethasone resulted in a reduced production of IL-6 in the head kidney [42], while in rainbow trout cortisol implantation down-regulated gene expression of *il-1β, tnfα* and complement component *C3* in the head kidney [43].

Fast et al. [44] studied the effects of both short- (a single 15 s out of water) and long-term (4 weeks of daily handling 15 s out of the water) stress on *il-1β* gene expression in HK macrophages of Atlantic salmon. They found that stress up-regulated *il-1β* gene expression in unstimulated cells, while in LPS-stimulated cells from stressed animals a significantly lower fold increase of *il-1β* was measured. During long-term stress the magnitude of the IL-1β response of isolated HK macrophages to an LPS stimulation was reduced up to 90% in stressed. A reduced sensitivity to stimulation was also observed in head kidney macrophages isolated from Atlantic salmon that were exposed to chronic hypoxic stress. Upon *ex vivo* poly I:C-stimulation these cells showed lower gene expression of the anti-viral IFNα and Mx [45]. Furthermore, utilizing the zebrafish tail amputation model, very recently Xie et al. [46] found that the synthetic glucocorticoid beclomethasone attenuated almost all wounding-induced changes in gene expression and as a consequence inhibited macrophage differentiation towards an M1 phenotype.

Also in mammals stress affects the macrophage activity/polarization. For example, in mice, restraint stress changed the phenotype of macrophages located at the wound site. Upon stress, gene expression of classical polarization markers: CXCL10 and CCL5, were down-regulated; while stress induced an up-regulation of IL10 gene expression [47]. Moreover, in mice under acute cold stress, LPS-stimulated macrophages showed an up-regulation of IL-10 [48]. Also monocytes, isolated from peripheral blood of patients intravenously injected with cortisol, exhibited increased gene expression of the chemokine receptors *ccr2* (marker of M2c polarization) and *cx3cr1* (marker of anti-inflammatory circulating monocytes) [49].

In the RAW264.7 cell line, stress induced by hypoxia or by stimulation with dexamethasone, increased the expression of glucocorticoid-induced leucine zipper (GILZ) while GILZ siRNA increased the expression of pro-inflammatory cytokines (*il-1β, tnfα, il-6*). These studies were confirmed *in vivo* where GILZ expression rose in rat spleen and lungs after hypoxia and/or dexamethasone injection [50]. Interestingly, increased GILZ expression was observed in monocytes/macrophages upon stimulation with cytokines involved in M2 macrophage polarization: IL-4, IL-10 and IL-13 [51,52]. Most probably, activation of glucocorticoid receptors leads to the inhibition of proinflammatory

genes associated with the transcription factors NFκB and AP1, while GILZ leads to increased expression of anti-inflammatory genes [51–53]. Also, in zebrafish embryos, glucocorticoids caused up-regulation of *gilz* expression [54,55], while in a tail amputation model of zebrafish larvae, the synthetic glucocorticoid - beclomethasone inhibited expression of over 80% of the immune related genes (e.g: *mmp9, cxcl-c1c, il-12a*). This phenomenon was not observed in GR mutant larvae [56].

Altogether, our results show that HK- and TK-derived monocytes/macrophages differ in their response to cortisol and stress. Both factors direct HK, but not TK macrophages to the path of alternative polarization It should nonetheless be noted that the M2 status can be attained in macrophages either by down-regulation of M1 markers, as was observed *in vitro* in HK cells treated with LPS + cortisol, or by up-regulation of M2 markers as observed in HK macrophages from stressed fish. The higher adeptness of HK monocytes/macrophages to polarize in the alternative direction upon stress/cortisol might be linked to a possibly higher paracrine exposure to locally produced stress hormones.

Our findings reveal that, like in mammals, also in fish glucocorticoids form important stimulators of alternative macrophage polarization. Therefore, also in fish, stress hormones released during challenging circumstances, will affect the inflammatory response and the process of tissue regeneration by actively controlling macrophage polarization. Especially farming conditions at high density therefore may form an extra risk. This circumstance of high infection rate combined with the impaired inflammatory response due to M2 polarization, caused by crowding stress, will facilitate disease propagation.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.08.064.

## References

[1] F.O. Martinez, L. Helming, S. Gordon, Alternative activation of macrophages: an immunologic functional perspective, Annu. Rev. Immunol. 27 (2009) 451–483, https://doi.org/10.1146/annurev.immunol.021908.132532.
[2] D.M. Mosser, The many faces of macrophage activation, J. Leukoc. Biol. 73 (2003) 209–212, https://doi.org/10.1189/jlb.0602325.
[3] F.O. Martinez, S. Gordon, The M1 and M2 paradigm of macrophage activation: time for reassessment, F1000Prime Rep. 6 (2014) 13, https://doi.org/10.12703/P6-13.
[4] A. Sica, P. Larghi, A. Mancino, L. Rubino, C. Porta, M.G. Totaro, M. Rimoldi, S.K. Biswas, P. Allavena, A. Mantovani, Macrophage polarization in tumour progression, Semin. Cancer Biol. 18 (2008) 349–355, https://doi.org/10.1016/j.semcancer.2008.03.004.
[5] A. Mantovani, A. Sica, S. Sozzani, P. Allavena, A. Vecchi, M. Locati, The chemokine system in diverse forms of macrophage activation and polarization, Trends Immunol. 25 (2004) 677–686, https://doi.org/10.1016/j.it.2004.09.015.
[6] T. Rőszer, Understanding the mysterious M2 macrophage through activation markers and effector mechanisms, Mediat. Inflamm. 2015 (2015) 1–16, https://doi.org/10.1155/2015/816460.
[7] D.M. Mosser, J.P. Edwards, Exploring the full spectrum of macrophage activation, Nat. Rev. Immunol. 8 (2008) 958–969, https://doi.org/10.1038/nri2448.
[8] S. Gordon, F.O. Martinez, Alternative activation of macrophages: mechanism and functions, Immunity 32 (2010) 593–604, https://doi.org/10.1016/j.immuni.2010.05.007.
[9] A.P. Gobert, K.T. Wilson, Editorial: Orchestration of macrophage polarization by polyamines, 91 (2012) 677–679, https://doi.org/10.1189/jlb.0112047.
[10] J.A.J. Arts, E.J. Tijhaar, M. Chadzinska, H.F.J. Savelkoul, B.M.L. Verburg-van Kemenade, Functional analysis of carp interferon-γ: evolutionary conservation of classical phagocyte activation, Fish Shellfish Immunol. 29 (2010) 793–802, https://doi.org/10.1016/j.fsi.2010.07.010.
[11] M. Joerink, C.M.S. Ribeiro, R.J.M. Stet, T. Hermsen, H.F.J. Savelkoul, G.F. Wiegertjes, Head kidney-derived macrophages of common carp (*Cyprinus carpio* L.) show plasticity and functional polarization upon differential stimulation, J. Immunol. 177 (2006) 61–69, https://doi.org/10.4049/jimmunol.177.1.61.
[12] K. Mao, W. Chen, Y. Mu, J. Ao, X. Chen, Identification of two IL-4/13 homologues in large yellow croaker (*Larimichthys crocea*) revealed their similar roles in inducing alternative activation of monocytes/macrophages, Fish Shellfish Immunol. 80 (2018) 180–190, https://doi.org/10.1016/j.fsi.2018.06.002.
[13] J.W. Hodgkinson, C. Fibke, M. Belosevic, Recombinant IL-4/13A and IL-4/13B induce arginase activity and down-regulate nitric oxide response of primary goldfish (*Carassius auratus* L.) macrophages, Dev. Comp. Immunol. 67 (2017) 377–384, https://doi.org/10.1016/j.dci.2016.08.014.
[14] M. Nguyen-Chi, B. Laplace-Builhe, J. Travnickova, P. Luz-Crawford, G. Tejedor, Q.T. Phan, I. Duroux-Richard, J. Levraud, K. Kissa, G. Lutfalla, C. Jorgensen, F. Djouad, Identification of polarized macrophage subsets in zebrafish, Elife 4 (2015) e07288, , https://doi.org/10.7554/eLife.07288.
[15] L.A. Moraes, S. Kar, S.L. Foo, T. Gu, Y.Q. Toh, P.B. Ampomah, K. Sachaphibulkij, G. Yap, O. Zharkova, H.M. Lukman, A.M. Fairhurst, A.P. Kumar, L.H.K. Lim, Annexin-A1 enhances breast cancer growth and migration by promoting alternative macrophage polarization in the tumour microenvironment, Sci. Rep. 7 (2017) 1–12, https://doi.org/10.1038/s41598-017-17622-5.
[16] V. Ferlazzo, P. D'Agostino, S. Milano, R. Caruso, S. Feo, E. Cillari, L. Parente, Anti-inflammatory effects of annexin-1: stimulation of IL-10 release and inhibition of nitric oxide synthesis, Int. Immunopharmacol. 3 (2003) 1363–1369, https://doi.org/10.1016/S1567-5769(03)00133-4.
[17] B.M.L. Verburg-van Kemenade, N. Cohen, M. Chadzinska, Neuroendocrine-immune interaction: evolutionarily conserved mechanisms that maintain allostasis in an ever-changing environment, Dev. Comp. Immunol. 66 (2017) 2–23, https://doi.org/10.1016/j.dci.2016.05.015.
[18] B.M.L. Verburg-van Kemenade, A. Groeneveld, B.T.T.M. van Rens, J.H.W.M. Rombout, Characterization of macrophages and neutrophilic granulocytes grom the pronephros of carp (*Cyprinus carpio*), J. Exp. Biol. 187 (1994) 143–158 http://www.ncbi.nlm.nih.gov/pubmed/9317515.
[19] L. Pijanowski, B.M.L. Verburg-van Kemenade, I. Irnazarow, M. Chadzinska, Stress-induced adaptation of neutrophilic granulocyte activity in K and R3 carp lines, Fish Shellfish Immunol. 47 (2015) 886–892, https://doi.org/10.1016/j.fsi.2015.10.030.
[20] K.M. Dubowski, An o-toluidine method for body-fluid glucose determination, Clin. Chem. 8 (1962) 215–235.
[21] M.W. Pfaffl, A new mathematical model for relative quantification in real-time RT-PCR, Nucleic Acids Res. 29 (2001) 45e–45, https://doi.org/10.1093/nar/29.9.e45.
[22] M. Chadzinska, H.F.J. Savelkoul, B.M.L. Verburg-van Kemenade, Morphine affects the inflammatory response in carp by impairment of leukocyte migration, Dev. Comp. Immunol. 33 (2009) 88–96, https://doi.org/10.1016/j.dci.2008.07.004.
[23] I.M. Corraliza, M.L. Campo, G. Soler, M. Modolell, Determination of arginase activity in macrophages: a micromethod, J. Immunol. Methods 174 (1994) 231–235, https://doi.org/10.1016/0022-1759(94)90027-2.
[24] M. Joerink, H.F.J. Savelkoul, G.F. Wiegertjes, Evolutionary conservation of alternative activation of macrophages: structural and functional characterization of arginase 1 and 2 in carp (*Cyprinus carpio* L.), Mol. Immunol. 43 (2006) 1116–1128, https://doi.org/10.1016/j.molimm.2005.07.022.
[25] H.D. Brightbill, S.E. Plevy, R.L. Modlin, S.T. Smale, A prominent role for Sp1 during lipopolysaccharide- mediated induction of the IL-10 promoter in macrophages, J. Immunol. 164 (2000) 1940–1951, https://doi.org/10.4049/jimmunol.164.4.1940.
[26] M. Goldsmith, D. Avni, O. Ernst, Y. Glucksam, G. Levy-Rimler, M.M. Meijler, T. Zor, Synergistic IL-10 induction by LPS and the ceramide-1-phosphate analog PCERA-1 is mediated by the cAMP and p38 MAP kinase pathways, Mol. Immunol. 46 (2009) 1979–1987, https://doi.org/10.1016/j.molimm.2009.03.009.
[27] W. Ma, W. Lim, K. Gee, S. Aucoin, D. Nandan, M. Kozlowski, F. Diaz-Mitoma, A. Kumar, The p38 mitogen-activated kinase pathway regulates the human interleukin-10 promoter via the activation of Sp1 transcription factor in lipopolysaccharide-stimulated human macrophages, J. Biol. Chem. 276 (2001) 13664–13674, https://doi.org/10.1074/jbc.M011157200.
[28] H. Chanteux, A.C. Guisset, C. Pilette, Y. Sibille, LPS induces IL-10 production by human alveolar macrophages via MAPKinases- and Sp1-dependent mechanisms, Respir. Res. 8 (2007) 1–10, https://doi.org/10.1186/1465-9921-8-71.
[29] W.W. Wang, C.P. Jenkinson, J.M. Griscavage, R.M. Kern, N.S. Arabolos, R.E. Byrns, S.D. Cederbaum, L.J. Ignarro, Co-induction of arginase and nitric oxide synthase in murine macrophages activated by lipopolysaccharide, Biochem. Biophys. Res. Commun. 210 (1995) 1009–1016, https://doi.org/10.1006/bbrc.1995.1757.
[30] Y. Jin, Y. Liu, L.D. Nelin, Extracellular signal-regulated kinase mediates expression of arginase II but not inducible nitric-oxide synthase in lipopolysaccharide-stimulated macrophages, J. Biol. Chem. 290 (2015) 2099–2111, https://doi.org/10.1074/jbc.M114.599985.
[31] E. Holen, S. Winterthun, Z.Y. Du, A.V. Krøvel, Inhibition of p38 MAPK during cellular activation modulate gene expression of head kidney leukocytes isolated from Atlantic salmon (*Salmo salar*) fed soy bean oil or fish oil based diets, Fish Shellfish Immunol. 30 (2011) 397–405, https://doi.org/10.1016/j.fsi.2010.11.017.
[32] S. MacKenzie, D. Iliev, C. Liarte, H. Koskinen, J.V. Planas, F.W. Goetz, H. Mölsä, A. Krasnov, L. Tort, Transcriptional analysis of LPS-stimulated activation of trout (*Oncorhynchus mykiss*) monocyte/macrophage cells in primary culture treated with cortisol, Mol. Immunol. 43 (2006) 1340–1348, https://doi.org/10.1016/j.molimm.2005.09.005.
[33] D.-C. Zhang, Y.-Q. Shao, Y.-Q. Huang, S.-G. Jiang, Cloning, characterization and expression analysis of interleukin-10 from the zebrafish (*Danio rerio*), BMB Rep. 38 (2005) 571–576, https://doi.org/10.5483/BMBRep.2005.38.5.571.
[34] R. Savan, D. Igawa, M. Sakai, Cloning, characterization and expression analysis of interleukin-10 from the common carp, *Cyprinus carpio* L. Eur. J. Biochem. 270 (2003) 4647–4654, https://doi.org/10.1046/j.1432-1033.2003.03854.x.
[35] Z.J. Yang, C.H. Li, J. Chen, H. Zhang, M.Y. Li, J. Chen, Molecular characterization of

an interleukin-4/13B homolog in grass carp (*Ctenopharyngodon idella*) and its role in fish against Aeromonas hydrophila infection, Fish Shellfish Immunol. 57 (2016) 136–147, https://doi.org/10.1016/j.fsi.2016.08.022.
[36] M. Chadzinska, K.M. Leon-Kloosterziel, B. Plytycz, B.M.L. Verburg-van Kemenade, *In vivo* kinetics of cytokine expression during peritonitis in carp: evidence for innate and alternative macrophage polarization, Dev. Comp. Immunol. 32 (2008) 509–518, https://doi.org/10.1016/j.dci.2007.08.008.
[37] J. Dong, J. Li, L. Cui, Y. Wang, J. Lin, Y. Qu, H. Wang, Cortisol modulates inflammatory responses in LPS-stimulated RAW264.7 cells via the NF-κB and MAPK pathways, BMC Vet. Res. 14 (2018) 30, https://doi.org/10.1186/s12917-018-1360-0.
[38] J.P. Saeij, L.B. Verburg-van Kemenade, W.B. van Muiswinkel, G.F. Wiegertjes, Daily handling stress reduces resistance of carp to *Trypanoplasma borreli: in vitro* modulatory effects of cortisol on leukocyte function and apoptosis, Dev. Comp. Immunol. 27 (2003) 233–245, https://doi.org/10.1016/S0145-305X(02)00093-9.
[39] E.H. Stolte, S.B. Nabuurs, N.R. Bury, A. Sturm, G. Flik, H.F.J. Savelkoul, B.M.L. Verburg-van Kemenade, Stress and innate immunity in carp: corticosteroid receptors and pro-inflammatory cytokines, Mol. Immunol. 46 (2008) 70–79, https://doi.org/10.1016/j.molimm.2008.07.022.
[40] A.R. Khansari, D. Parra, F.E. Reyes-López, L. Tort, Cytokine modulation by stress hormones and antagonist specific hormonal inhibition in rainbow trout (*Oncorhynchus mykiss*) and gilthead sea bream (*Sparus aurata*) head kidney primary cell culture, Gen. Comp. Endocrinol. 250 (2017) 122–135, https://doi.org/10.1016/j.ygcen.2017.06.005.
[41] C.C. Wu, J.D. Croxtall, M. Perretti, C.E. Bryant, C. Thiemermann, R.J. Flower, J.R. Vane, Lipocortin 1 mediates the inhibition by dexamethasone of the induction by endotoxin of nitric oxide synthase in the rat, Proc. Natl. Acad. Sci. U.S.A. 92 (1995) 3473–3477, https://doi.org/10.1073/pnas.92.8.3473.
[42] R. Kumar, K.P. Joy, Stress hormones modulate lipopolysaccharide stimulation of head kidney interleukin-6 production in the catfish *Heteropneustes fossilis: In vivo* and *in vitro* studies, Gen. Comp. Endocrinol. (2019), https://doi.org/10.1016/j.ygcen.2019.01.005 0–1.
[43] R. Cortés, M. Teles, R. Trídico, L. Acerete, L. Tort, Effects of cortisol administered through slow-release implants on innate immune responses in rainbow trout (*Oncorhynchus mykiss*), Int. J. Genom. 2013 (2013) 1–7, https://doi.org/10.1155/2013/619714.
[44] M.D. Fast, S. Hosoya, S.C. Johnson, L.O.B. Afonso, Cortisol response and immune-related effects of Atlantic salmon (*Salmo salar* Linnaeus) subjected to short- and long-term stress, Fish Shellfish Immunol. 24 (2008) 194–204, https://doi.org/10.1016/j.fsi.2007.10.009.
[45] B.O. Kvamme, K. Gadan, F. Finne-Fridell, L. Niklasson, H. Sundh, K. Sundell, G.L. Taranger, Ø. Evensen, Modulation of innate immune responses in Atlantic salmon by chronic hypoxia-induced stress, Fish Shellfish Immunol. 34 (2013) 55–65, https://doi.org/10.1016/j.fsi.2012.10.006.
[46] Y. Xie, S. Tolmeijer, J.M. Oskam, T. Tonkens, A.H. Meijer, M.J.M. Schaaf, Glucocorticoids Inhibit Macrophage Differentiation towards a Pro- Inflammatory Phenotype upon Wounding without Affecting Their Migration, (2019), https://doi.org/10.1242/dmm.037887.
[47] S.D. Tymen, I.G. Rojas, X. Zhou, Z.J. Fang, Y. Zhao, P.T. Marucha, Restraint stress alters neutrophil and macrophage phenotypes during wound healing, Brain Behav. Immun. 28 (2013) 207–217, https://doi.org/10.1016/j.bbi.2012.07.013.
[48] R. Sesti-Costa, G.C. Baccan, S. Chedraoui-Silva, B. Mantovani, Effects of acute cold stress on phagocytosis of apoptotic cells: the role of corticosterone, Neuroimmunomodulation 17 (2010) 79–87, https://doi.org/10.1159/000258690.
[49] M.P. Yeager, P.A. Pioli, J. Collins, F. Barr, S. Metzler, B.D. Sites, P.M. Guyre, Glucocorticoids enhance the *in vivo* migratory response of human monocytes, Brain Behav. Immun. 54 (2016) 86–94, https://doi.org/10.1016/j.bbi.2016.01.004.
[50] Y. Wang, Y.-Y. Ma, X.-L. Song, H.-Y. Cai, J.-C. Chen, L.-N. Song, R. Yang, J. Lu, Upregulations of glucocorticoid-induced leucine zipper by hypoxia and glucocorticoid inhibit proinflammatory cytokines under hypoxic conditions in macrophages, J. Immunol. 188 (2012) 222–229, https://doi.org/10.4049/jimmunol.1002958.
[51] D. Berrebi, S. Bruscoli, N. Cohen, A. Foussat, G. Migliorati, L. Bouchet-Delbos, M.C. Maillot, A. Portier, J. Couderc, P. Galanaud, M. Peuchmaur, C. Riccardi, D. Emilie, Synthesis of glucocorticoid-induced leucine zipper (GILZ) by macrophages: an anti-inflammatory and immunosuppressive mechanism shared by glucocorticoids and IL-10, Blood 101 (2003) 729–738, https://doi.org/10.1182/blood-2002-02-0538.
[52] J. Hoppstädter, S.M. Kessler, S. Bruscoli, H. Huwer, C. Riccardi, A.K. Kiemer, Glucocorticoid-induced leucine zipper: a critical factor in macrophage endotoxin tolerance, J. Immunol. 194 (2015) 6057–6067, https://doi.org/10.4049/jimmunol.1403207.
[53] S. Ronchetti, G. Migliorati, C. Riccardi, GILZ as a mediator of the anti-inflammatory effects of glucocorticoids, Front. Endocrinol. (Lausanne). 6 (2015) 1–6, https://doi.org/10.3389/fendo.2015.00170.
[54] R.A. Willi, N. Salgueiro-González, G. Carcaiso, K. Fent, Glucocorticoid mixtures of fluticasone propionate, triamcinolone acetonide and clobetasol propionate induce additive effects in zebrafish embryos, J. Hazard Mater. 374 (2019) 101–109, https://doi.org/10.1016/j.jhazmat.2019.04.023.
[55] R.A. Willi, N. Salgueiro-González, S. Faltermann, T. Hettich, K. Fent, Environmental glucocorticoids corticosterone, betamethasone and flumethasone induce more potent physiological than transcriptional effects in zebrafish embryos, Sci. Total Environ. 672 (2019) 183–191, https://doi.org/10.1016/j.scitotenv.2019.03.426.
[56] A. Chatzopoulou, J.P.M. Heijmans, E. Burgerhout, N. Oskam, H.P. Spaink, A.H. Meijer, M.J.M. Schaaf, Glucocorticoid-induced attenuation of the inflammatory response in zebrafish, Endocrinology 157 (2016) 2772–2784, https://doi.org/10.1210/en.2015-2050.