Early life glucocorticoid exposure modulates immune function in zebrafish (*Danio rerio*) larvae

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Abstract (number of words: 239)

In this study we have assessed the effects of increased cortisol levels during early embryonic development on immune function in zebrafish (*Danio rerio*) larvae. Fertilized eggs were exposed to either a cortisol-containing, a dexamethasone-containing (to stimulate the glucocorticoid receptor selectively) or a control medium for 6 hours post-fertilisation (0-6 hpf). First, we measured baseline expression of a number of immune-related genes (*socs3a*, *mpeg1.1*, *mpeg1.2* and *irg1l*) 5 days post-fertilisation (dpf) in larvae of the AB and TL strain to assess the effectiveness of our exposure procedure and potential strain differences. Cortisol and dexamethasone strongly up-regulated baseline expression of these genes independent of strain. The next series of experiments were therefore carried out in larvae of the AB strain only. We measured neutrophil/macrophage recruitment following tail fin amputation (performed at 3 dpf) and phenotypical changes as well as survival following LPS-induced sepsis (150 μg/ml; 4-5 dpf). Dexamethasone, but not cortisol, exposure at 0-6 hpf enhanced neutrophil recruitment 4 hours post tail fin amputation. Cortisol and dexamethasone exposure at 0-6 hpf led to a milder phenotype (e.g. less tail fin damage) and enhanced survival following LPS challenge compared to control exposure. Gene-expression analysis showed accompanying differences in transcript abundance of *tlr4bb, cxcr4a, myd88*, *il1β and il10*. These data show that early-life exposure to cortisol, which may be considered to be a model or proxy of maternal stress, induces an adaptive response to immune challenges, which seems mediated via the glucocorticoid receptor.

36 **Abbreviations used in the manuscript**

37 **ANOVA**: analysis of variance; **GR**: glucocorticoid receptor; **dpf**: days post-fertilisation; **GFP**: green
38 fluorescent protein; **hpa**: hours post-amputation; **hpf**: hours post-fertilisation; **HPI-axis**: hypothalamus
39 – pituitary - interrenal axis; **IL**: interleukine; **KMO**: Kaiser-Meyer-Olkin; **LPS**: lipopolysaccharide;
40 **MANOVA**: multivariate analysis of variance; **MR**: mineralocorticoid receptor; **ns**: not significant; **PBS:**
41 phosphate buffered saline; **qPCR**: quantitative polymerase chain reaction; **SEM**; standard error of the
42 mean; **TLR**: Toll-like receptor; **Tukey HSD**: Tukey Honestly Significant Difference

1. Introduction

In teleosts, like zebrafsh (*Danio rerio)*, cortisol is the main endogenous corticosteroid, which is secreted when individuals perceive situations as stressful (Wendelaar Bonga, 1997). Like in other vertebrate species, cortisol binds in teleosts to the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR), which affect the transcription rates of genes following ligand binding (Alderman and Bernier, 2009; Alsop and Vijayan, 2008; Nesan and Vijayan, 2013). Since the GR has a lower affinity for cortisol than the MR, the GR mediates the actions of cortisol during stress, which involves optimizing energy expenditure by tuning the balance between and within physiological systems, like an organism's metabolism and its immune, cardiovascular and central nervous system (e.g. Gorissen and Flik, 2016; Nesan and Vijayan, 2013; Wendelaar Bonga, 1997). Following long-term exposure to stress, baseline levels of cortisol are increased, reflecting the allostatic load that the environment imposes on an organism (Gorissen and Flik, 2016; McEwen and Wingfield, 2003). Cortisol has been shown to signal through the GR already during the very early stages of embryonic development; in oocytes maternally deposited cortisol and GR mRNA are present (Pikulkaew *et al.*, 2010, 2011; Wilson *et al.*, 2013). These cortisol levels may reflect the allostatic load that the mothers experience in their environment and it has therefore been hypothesized that these deposited cortisol levels are important for preparing the offspring for the expected allostatic load that larvae will encounter in the prevailing environment, thereby programming their cortisol secretion and the functioning of physiological systems to meet expected demands (Nesan and Vijayan, 2013; van den Bos *et al.*, 2019). While in previous studies we focussed on the effects of cortisol exposure (between 0 and 6 hours post fertilisation (hpf)) on vigilance-related behaviour, baseline cortisol levels and metabolism in larvae (van den Bos *et al.*, 2019; van den Bos, 2019) in the present study we focussed on the functioning of the immune system, in particular the innate immune system on which zebrafish larvae rely (Meijer and Spaink, 2011). Cortisol exposure may be considered to be a model or proxy of maternal stress (Best *et al.*, 2017; Nesan and Vijayan, 2012, 2016; van den Bos *et al.*, 2019).

Zebrafish is a highly suitable animal model to study early life events in the fields of biomedical research, behavioural biology and eco-toxicology (e.g. Champagne *et al.*, 2010; Dai *et al.*, 2013; Nesan and Vijayan, 2013; Steenbergen *et al.*, 2011; Stewart *et al.*, 2014). Fertilized eggs develop into independently feeding larvae outside the mother, without parental care, and can easily be maintained under different experimental conditions as well as pharmacologically manipulated. In zebrafish, it has

been demonstrated that mothers deposit cortisol and GR mRNA in oocytes (Pikulkaew *et al.*, 2010, 2011; Wilson *et al.*, 2013). These cortisol levels decrease over the first 24 hours post fertilisation, after which zygotes start to produce cortisol by the then developing interrenal cells (Liu, 2007; Pikulkaew *et al.*, 2010, 2011; Wilson *et al.*, 2013). After hatching (48-72 hpf) pituitary control over interrenal cortisol production starts and it takes another 4-5 days before the hypothalamus-pituitary-interrenal (HPI) axis is fully functionally mature (Alderman and Bernier, 2009; Alsop and Vijayan, 2008). Maternal GR mRNA is present during the first 6 hpf, and at 8-9 hpf zygotic expression of the GR commences, while the MR mRNA production starts at 24 hpf (Alsop and Vijayan, 2008; Pikulkaew *et al.*, 2010, 2011).

In several studies the effect of cortisol exposure during early embryonic stages has been investigated in zebrafish. In these studies cortisol levels were increased by injection of cortisol into the yolk of one-cell stage embryos (e.g. Best *et al.*, 2017; Nesan and Vijayan, 2012, 2016) or through addition of cortisol to the medium (Hartig *et al.*, 2016; van den Bos *et al*., 2019). These studies showed that, as a result of the cortisol exposure during early embryonic stages, larval baseline levels of cortisol were increased (Best *et al.*, 2017; Hartig *et al.*, 2016; Nesan and Vijayan, 2012; van den Bos *et al.*, 2019). For example, in a recent study, we have demonstrated that cortisol exposure between 0 and 6 hpf increased baseline cortisol levels 5 days post fertilisation (dpf) and this effect was stronger in larvae from the AB strain than in larvae from the TL strain (van den Bos *et al*., 2019).

Exposure to cortisol (0-5 dpf) has been shown to lead to an enhanced expression of immune-related genes in zebrafish larvae at 5 dpf (Hartig *et al*, 2016), suggesting that early cortisol exposure increases the activity of the immune system. In the present study, we have first measured the expression of a selected number of these up-regulated genes at 5 dpf (*socs3a*, *mpeg1.1*, *mpeg1.2*, *irg1l*) following 0-6 hpf exposure to cortisol in zebrafish larvae of the AB strain to assess whether our method produces similar effects. Tüpfel long-fin (TL) is another widely used zebrafish strain next to AB and is characterized by spots rather than stripes as well as long fins rather than short fins. In previous studies we have observed consistent differences between larvae of the AB strain and larvae of the TL strain at the level of both HPI-axis activity and behaviour (van den Bos *et al*., 2017a, 2017b, 2019a, 2020). We have attributed these differences to the mutation in the connexin 41.8 gene that leads to spots (for discussion: see van den Bos *et al.*, 2020). Measuring the expression of these genes in larvae of the TL strain next to larvae in the AB strain may therefore reveal how robust our findings are.

102 Finally, to assess the role of the GR in more detail, we exposed fertilized eggs 0-6 hpf to
103 dexamethasone, a specific GR agonist (Rupprecht *et al.*, 1993).
104 To functionally assess the activity of the immune system following early life exposure to
105 cortisol or dexamethasone, we used two experimental models for immune activation. First, we used
106 the tail fin amputation assay. This is a well-established model in which amputation of the tail triggers
107 expression of many pro-inflammatory molecules and the recruitment of innate immune cells
108 (neutrophils and macrophages) towards the wounded area (Hall *et al.*, 2014; Renshaw *et al.*, 2006;
109 Roehl, 2018). The tail fin amputation assay was performed using the double transgenic fish line
110 *Tg(mpx:GFP/mpeg1:mCherry-F)* (Bernut *et al.*, 2014; Renshaw *et al.*, 2006). Recruitment of
111 neutrophils and macrophages was determined following tail fin amputation in larvae at 3 dpf
112 (Chatzopoulou *et al.,* 2016; Xie *et al.*, 2019). Second, we used a sepsis model, which involved a
113 challenge with lipopolysaccharide (LPS), the membrane component of Gram-negative bacteria, in 4
114 dpf larvae. We measured survival, phenotypical changes, and the expression of a series of LPS-
115 responsive genes (Dios *et al.*, 2014; Hsu *et al.*, 2018; Novoa *et al.*, 2009; Philip *et al.*, 2017).
116
117 **2. Materials and Methods**
118 **2.1. Subjects, spawning and care**
119 Housing conditions and breeding procedures were similar as those reported in van den Bos *et al.*
120 (2017a, 2017b, 2019; AB and Tüpfel long-fin (TL) strains) or Xie *et al.* (2019; the double transgenic
121 fish line *Tg(mpx:GFP/mpeg1:mCherry-F).* They were kept in recirculation systems (~28°C) under a
122 14h:10h light-dark cycle and fed twice daily.
123 Breeding started at least one hour after the last feeding of zebrafish (>16:00 h). Males and
124 females of the AB or TL strain were placed in a zebrafish breeding tank, separated by a partitioning
125 wall, with water of ~28°C. After turning on the lights the next morning, the partitioning wall was
126 removed and tanks were placed at a slight angle, such that the fish had the possibility to move into
127 shallow water to spawn.
128
129 **2.2. Cortisol/dexamethasone exposure during early embryonic development (0-6 hpf)**
130 Cortisol (hydrocortisone; Sigma-Aldrich, Zwijndrecht, the Netherlands) and dexamethasone (Sigma-
131 Aldrich, Zwijndrecht, the Netherlands) were dissolved in 96% ethanol in the required stock solution

132 concentrations and stored at -20°C. From these stock solutions media with the appropriate
133 concentration were freshly prepared for each experiment (Althuizen, 2018; van den Bos *et al.*, 2019):
134 cortisol-containing medium: 400 µg/l cortisol (1.1 µM), 0.4 ml/l 96% ethanol (0.04% v/v), 5 mM NaCl,
135 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$ in dH2O; dexamethasone-containing medium: 430
136 µg/l dexamethasone (1.1 µM), 0.4 ml/l 96% ethanol (or 1 ml/l 96% ethanol (0.1% v/v)); depending on
137 the specific experiment), 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$ in dH2O.
138 Control medium consisted of: 0.4 ml/l 96% ethanol (or 1 ml/l 96% ethanol; depending on the specific
139 experiment), 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$ in dH2O.
140 Directly following spawning and fertilization, eggs were collected and randomly assigned to
141 Petri dishes filled with either cortisol-containing, dexamethasone-containing or control medium. Within
142 1-1.5 hpf Petri dishes were placed in an incubator set at 28.5 °C (300–350 lux). Eggs were exposed to
143 these solutions for 6 hrs. It has been shown that both cortisol and dexamethasone diffuse inside the
144 eggs in this period (Steenbergen *et al.*, 2017). In addition, we have shown this procedure to be
145 effective in eliciting changes in physiology and behaviour at 5 dpf (Althuizen, 2018; van den Bos *et al.*,
146 2019).
147 Following this, cortisol-containing, dexamethasone-containing and control media were
148 replaced by E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, 3 ml/l 0.01%
149 (w/v) methylene blue in dH2O). Embryos were rinsed twice in E3 medium to ensure that the original
150 treatment media were completely removed. Petri dishes were returned to the incubator allowing the
151 embryos to develop further (28.5°C; 14h:10h light-dark period (lights on: 09.00h – 23.00h); light phase:
152 300–350 lux; dark phase; 0 lux). At 1 and 4 dpf E3 medium was refreshed and unfertilized eggs, dead
153 eggs/embryos/larvae and chorions were removed from the dishes.
154 All experiments were carried out in accordance with the Dutch Experiments on Animals Act
155 (http://wetten.overheid.nl/BWBR0003081/2014-12-18), the European guidelines for animal
156 experiments (Directive 2010/63/EU; http://eur-lex.europa.eu/legal-content/NL/TXT/HTML/?uri=
157 CELEX:32010L0063) and institutional regulations (Radboud University or Leiden University). Larvae
158 were euthanized by placing them in ice slurry for at least 20 minutes followed by adding bleach to the
159 slurry. In case of anaesthesia 0.01% 2-phenoxyethanol (Sigma Aldrich, Zwijndrecht, the Netherlands;
160 experiments Radboud University) or 0.02% buffered aminobenzoic acid ethyl ester (tricaine; Sigma
161 Aldrich, Zwijndrecht, the Netherlands; experiments Leiden University) was used.

162

163 **2.3 Baseline gene expression analysis.**

164 Following 0-6 hpf exposure to the different media, larvae of the AB and TL strain were sampled for
165 gene expression at 5 dpf between 16:00 hrs and 19:00 hrs (van den Bos *et al.*, 2019; experimental
166 time-line: figure 1). Genes of interest were: *socs3a*, *mpeg1.1*, *mpeg1.2* and *irg1l* (for primer
167 sequences of these genes: see van den Bos *et al.*, 2017a). Gene expression was determined by
168 qPCR analysis as described below.

169

170 **2.4 Tail fin amputation assay**

171 The tail fin amputation assay was performed using the double transgenic fish line
172 *Tg(mpx:GFP/mpeg1:mCherry-F;* Bernut *et al.*, 2014; Renshaw *et al.*, 2006). Three-day-old zebrafish
173 larvae were anaesthetized in E3 medium containing 0.02% buffered tricaine and loaded onto 2%
174 agarose-coated Petri dishes (experimental time-line: figure 1). Amputation was performed with a 1 mm
175 sapphire blade (World Precision Instruments, Friedberg, Germany) using a Leica M165C
176 stereomicroscope and a micromanipulator (Leica Microsystems BV, Amsterdam, the Netherlands; see
177 below, figure 3A). Larvae were fixed in 4% paraformaldehyde in PBS at 4 hours post amputation (hpa)
178 and stored overnight at 4°C. The following day larvae were washed twice for one minute and then
179 twice for five minutes in PBS containing 0.01% Tween 20 (Sigma Aldrich, Zwijndrecht, the
180 Netherlands).

181 A Leica M205FA fluorescence stereomicroscope supported by LAS software (version 4.12.0;
182 Leica Microsystems BV, Amsterdam, the Netherlands) was utilised to visualise the leukocytes.
183 Detection of neutrophils and macrophages was based on their fluorescent GFP and mCherry signals
184 respectively. To quantify cell migration towards the wounded area, cells within a distance of 200µm
185 from the amputation site were counted manually, as previously described (Xie *et al.*, 2019). Data
186 (numbers of migrated neutrophils and macrophages per individual) were pooled from three individual
187 experiments (n>10 per experiment), and the presented data are means (± SEM).

188

189 **2.5. LPS exposure**

190 First, we conducted a pilot study to assess the optimal LPS dose, exposure duration and parameters
191 to be measured (protocols adapted from: Dios *et al.*, 2014; Hsu *et al.*, 2018; Novoa *et al.*, 2009; Philip

192 *et al.*, 2017). Incubation for 30 minutes in 150 μg/ml LPS (B11.04; Sigma Aldrich, Zwijndrecht, the
193 Netherlands) was effective in eliciting a robust increase in *il1β* expression (assessed using qPCR
194 analysis), changes in tail fin morphology (swollen or damaged tails) and increased levels of reactive
195 oxygen species (ROS; measured by a fluorescent labelling method according to Philip *et al.*, 2017).
196 Hence, we used this dose in subsequent experiments.

197 Subsequently, two experiments were conducted. In both experiments 4 dpf larvae were
198 exposed to 150 μg/ml LPS (B11.04; Sigma Aldrich, Zwijndrecht, the Netherlands) in E2 medium or
199 control E2 medium for 30 minutes in Petri dishes (n=50 in 25 ml; experimental time-line: figure 1).
200 Following this exposure, LPS-containing medium or control E2 medium was replaced by fresh E2
201 medium (larvae were rinsed two times to ensure that the original media were removed). Larvae either
202 remained in the Petri dishes for sampling for gene expression at later time points or were transferred
203 individually to 24 wells plates (Greiner Bio-One BV, Alphen a/d Rijn, the Netherlands) for assessing
204 phenotypical changes and survival (volume per well: 1-1.5 ml). Six treatment groups were thus
205 created: 0-6 hpf control, cortisol or dexamethasone treatment, combined with either 4 dpf LPS or
206 control treatment (for 30 min).

207 In the first experiment the level of gene expression at 0 hr, 0.5 hr (i.e. directly following
208 exposure), 1 hr (i.e. 30 minutes after ending exposure) and 3 hrs (i.e. 2.5 hrs after ending exposure;
209 Novoa *et al.*, 2009) was determined by qPCR analysis as described below. Genes of interest (see
210 papers by: Hsu *et al.*, 2018; Kanwal *et al.*, 2013; Novoa *et al.*, 2009; Philip *et al.*, 2017) were genes
211 encoding proteins involved in barrier function of the vascular endothelium (*cldn5a*, *cldn2*, *oclnb*), Toll-
212 like receptors (*tlr2*, *tlr4ba*, *tlr4bb*, *tlr5a*, t*lr5b*), and regulators of the immune response (*il1β*, *il10*,
213 *myd88*, *cxcr4a, cxcr4b, ptpn6*). Primer sequences are listed in Table 1.

214 In addition, in the first experiment survival and phenotypical changes were determined at 0, 4
215 (i.e. 3.5 hrs after ending exposure) and 28 hrs (i.e. 27.5 hrs after ending exposure). Phenotypical
216 changes included (see Philip *et al.*, 2017): changes in tail fin morphology (normal, swollen (oedema)
217 or damaged), presence of heart oedema and changes in shape (straight or curved). In the second
218 experiment (that also served as replicate for the first experiment) survival and phenotypical changes
219 were measured at 0, 0.5, 1, 3, 6 and 24 hrs (i.e. before exposure, directly after exposure, 0.5 hrs after
220 ending exposure, 2.5 hrs after ending exposure, 5.5 hrs after ending exposure and 23.5 hrs after
221 ending exposure).

222

223 **2.6 Gene expression analysis**

224 For the assessment of gene expression levels by qPCR analysis, 3-5 larvae were transferred to a 2-ml
225 Eppendorf tube; thus, one sample contained material from 3-5 larvae. Residual medium was removed
226 with a pipette, tubes were snap frozen in liquid nitrogen, kept on ice during the sampling procedure,
227 and subsequently stored at -80 °C until total RNA extraction.

228 RNA isolation, RNA preparation, removal of genomic DNA from the samples and synthesis of
229 cDNA was performed according to the protocol described in van den Bos *et al.* (2017a). Total RNA
230 content of each sample was isolated. This was done by homogenising the tissue with 400 µl Trizol
231 reagent (Invitrogen, Carlsbad, USA) in a Grinding Mill (Retsch GmbH, Germany) for 20 s at 20 Hz.
232 After homogenisation, samples were kept at room temperature for 5 min. Next, 80 µl chloroform was
233 added and the solution was mixed by shaking for 15 s. Afterwards, samples were kept at room
234 temperature for 2 min. The samples were centrifuged at 14,000 rpm for 10 min in a cooled centrifuge
235 (4°C) and the aqueous phase of the samples was transferred to a new tube. To this phase, 200 µl
236 isopropanol was added and this solution was mixed well by inversion of the tube. The solution was
237 then stored at -20°C for 2 h and centrifuged afterwards for 15 min at 14,000 rpm in a cooled centrifuge
238 (4°C). The supernatant was decanted and the pellet washed with 500 µl 75% ethanol and centrifuged
239 10 min at 14,000 rpm in a cooled centrifuge (4°C). The supernatant was decanted, after which the
240 pellet was centrifuged for 5 s to remove all the remaining supernatant using a pipette. The pellet
241 containing the RNA was air-dried for 10 min at room temperature and afterwards dissolved in 100 µl
242 ice cold DEPC-treated dH2O. To this RNA solution, 10 µl 3M NaAc (pH 5.4) and 250 µl 100% ethanol
243 were added. The solution was mixed by inverting the tube and samples were stored for 2 h at -20°C.
244 Subsequently, the samples were centrifuged for 15 min at 14,000 rpm in a cooled centrifuge (4°C),
245 and the supernatant was decanted and the pellet washed washed as described earlier. Finally, the
246 RNA pellet was dissolved in 15 µl DEPC-treated dH2O. The concentration and quality of RNA in each
247 sample were measured using a nanodrop spectrophotometer at 260 nm wavelength (Nanodrop,
248 Wilmington, DE, USA).

249 Isolated RNA was treated with DNase to remove any (genomic) DNA from the sample; 400 ng
250 RNA was transferred into a PCR strip, and DEPC-treated dH2O was added to a volume of
251 8 µl. To this, 2 µl of DNase mix was added, containing 1 µl 10x DNase I reaction buffer and

1 µl (1 U/µl) amplification grade DNase I (both from Invitrogen, Carlsbad, USA). The resulting mix was incubated for 15 min at room temperature. Afterwards, 1 µl 25 mM EDTA was added to stop the DNase reaction and the reaction mix was incubated for 10 min at 65°C and returned on ice.

After the DNase treatment, samples were used to synthesize cDNA by the addition of 1 µl random primers (250 ng/µl), 1 µl 10 mM dNTP mix, 4 µl 5 x 1st strand buffer, 1 µl 0.1M DTT, 1 µl RNase inhibitor (10 U/µl), 0.5 µl Superscript II (reverse transcriptase) (200 U/µl) (all from Invitrogen, Carlsbad, USA) and 0.5 µl DEPC-treated dH2O. The resulting mix was incubated for 10 min at 25°C for annealing of the primers and then 50 min at 42°C for reverse transcription. Enzymes were hereafter inactivated by incubating samples at 70°C for 15 min. Finally, 80 µl dH2O was added to dilute the samples five times for the qPCR reaction.

To measure the relative gene expression in each sample, real-time qPCR was carried out for each gene of interest. For each qPCR reaction, 16 µl PCR mix (containing 10 µl SYBR green mix (2x) (BioRad, Hercules, USA), 0.6 µl forward and reverse gene-specific primer (10 µM) and 4.8 µl H2O) was added to 4 µl of cDNA. The qPCR reaction (3 min 95°C, 40 cycles of 15 s 95°C and 1 min 60°C) was carried out using a CFX 96 (BioRad, Hercules, USA) qPCR machine. Analysis of the data was carried out using a normalisation index of two reference genes (viz. *elongation factor alpha* (*elf1a*) and *ribosomal protein L13* (*rpl13*)) (Vandesompele *et al.*, 2002).

2.7. Statistics

For gene expression analyses, outliers were removed following Grubb's outlier test ($p \leq 0.01$). We explored the interrelationships of transcript abundance levels using Principal Component Analysis (PCA) with orthogonal rotation (Varimax rotation with Kaiser normalization; see van den Bos *et al.*, 2017b). In case of missing samples, data were excluded list-wise. The number of retained components was based on eigenvalues (>1) and visual inspection of the scree plot. The Kaiser-Meyer-Olkin (KMO) measure of sampling adequacy and Bartlett's test of sphericity were done to ensure that data obeyed analysis criteria; both are measures to assess whether the correlation matrix is suited for factor analysis (Budaev, 2010). Component scores were saved and used for further statistical analysis. The following component loading cut-off points were considered: ≤ -0.600 or ≥ 0.600 (Ferguson, 1989; Budaev, 2010).

281 For the basal gene expression values a two-way or three-way Analysis of Variance (ANOVA)
282 was run with treatment, strain or batch (where applicable) as independent factors. In the tail fin
283 amputation assay a Student's t-test was run on the number of neutrophils or macrophages at 4 hpa
284 comparing 0-6 hpf treatment groups (cortisol *versus* control; dexamethasone *versus* control).
285 In the LPS exposure experiment, for gene expression a multivariate analysis of variance (MANOVA)
286 was run (to account for multiple comparisons) followed by univariate analysis of variance (0-6 hpf
287 treatment and time as independent factors followed by post-hoc testing (Tukey HSD)). In addition per
288 time point a one-way ANOVA was run with 0-6 hpf treatment as factor followed by post-hoc testing
289 (Tukey HSD).
290 In the LPS exposure experiment differences in survival rate were assessed using the Kaplan-
291 Meijer procedure (Log Rank Mantel-Cox). Differences in phenotypical changes were compared using
292 Chi-square tests.
293 Significance was set at $p \leq 0.05$ and trends are indicated ($p \leq 0.10$) where appropriate; ns: not
294 significant: $p > 0.10$. Unless otherwise stated all p-values are two-tailed. All statistical analyses were
295 done using IBM SPSS version 23 (IBM, Armonk, NY, USA).
296
297 **3 Results**
298 **3.1 Baseline gene expression analysis**
299 Rather than analysing transcript abundance of different genes following glucocorticoid treatment
300 (cortisol or dexamethasone, 0-6 hpf) separately, we explored the effects of exposure through the
301 interrelationships of transcript abundance of genes using PCA.
302 A PCA for the 0-6 hpf cortisol treatment experiment revealed two components (figure 2A;
303 supplementary figure 1A shows the transcript abundance of individual genes). The KMO value was
304 sufficiently high (0.522) and Bartlett's test of sphericity was highly significant (Chi-square=17.707,
305 df=6, $p \leq 0.007$), indicating that the data were adequate for a PCA. The first component (explaining
306 44.0% of the variance) was comprised of *irg1l* (loading: 0.854) and *socs3a* (loading: 0.890); the
307 second component (explaining 28.9% of the variance) of *mpeg1.1* (loading: 0.890) and *mpeg1.2*
308 (loading: 0.890). Each component was analysed separately using ANOVA. Treatment (0-6 hpf) with
309 cortisol enhanced transcript abundance of genes in component 1 (*irg1l* and *socs3a*) independent of
310 strain (three-way ANOVA (strain, treatment, batch): treatment: $F(1,23)=6.477$, $p \leq 0.018$). Transcript

abundance was overall higher in TL than AB larvae (strain: $F(1,23)=7.023$, $p \leq 0.014$). Treatment (0-6 hpf) with cortisol enhanced transcript abundance of genes in component 2 (*mpeg1.1* and *mpeg1.2)* independent of strain (three-way ANOVA (strain, treatment, batch): treatment: $F(1,23)=7.660$, $p \leq 0.011$).

A PCA for the 0-6 hpf dexamethasone treatment experiment revealed only one component (figure 2B; supplementary figure 1B shows the transcript abundance of individual genes). The KMO was sufficiently high (0.626) and Bartlett's test of sphericity was highly significant (Chi-square=102.945, df=6, $p<0.001$), indicating that the data were adequate for a PCA. This component explained 76.6% of the variance. Loadings onto this component were: *irg1l (*0.867). *socs3a* (0.835), *mpeg1.1* (0.928) and *mpeg1.2* (0.868). Treatment with dexamethasone enhanced transcript abundance of genes independent of strain (two-way ANOVA (strain, treatment): treatment: $F(1,33)=6.745$, $p \leq 0.014$).

These data show that glucocorticoid treatment was effective in eliciting changes in baseline expression of (a selected set of) immune-related genes.

3.2 Tail fin amputation assay

Figure 3A shows the site of the tail fin amputation performed at 3 dpf. Neutrophil recruitment at 4 hpa was enhanced in the 0-6 hpf dexamethasone treatment group (Student's t-test: $t=2.917$, df=106, $p \leq 0.004$; figure 3C), but not in the 0-6 hpf cortisol treatment group (Student's t-test: $t=0.621$, df=89, ns; figure 3B), compared to the group treated with control medium at 0-6 hpf. No effects were found for macrophage recruitment for either 0-6 hpf cortisol treatment (Student's t-test: $t=0.923$, df=89, ns; figure 3B) or 0-6 hpf dexamethasone treatment (Student's t-test: $t=-1.095$, df=106, ns; figure 3C).

3.3 LPS exposure

Survival

In the LPS exposure experiments, we never observed any morphological changes or mortality in the 4 dpf control groups. Hence, we only present the data of the 4 dpf LPS-treated groups. Figure 3 shows the survival data of both LPS exposure experiments. Only 27.8% of the 0-6 hpf control-treated larvae survived in the first LPS exposure experiment (figure 4A), while 79.2% survived in the second LPS exposure experiment (figure 4B). In both LPS exposure experiments the number of larvae that

341 survived following glucocorticoid treatment 0-6 hpf appeared to be higher (Log Rank Mantel Cox; first
342 LPS exposure experiment: overall Chi-square=20.863, df=2, p<0.001; second LPS exposure
343 experiment: overall Chi-square=5.824, df=2, p≤0.054). Pair-wise comparison revealed that (1) in both
344 LPS exposure experiments survival was significantly higher in 0-6 hpf cortisol-treated than 0-6 hpf
345 control-treated larvae ((Log Rank Mantel Cox; Chi-square=14.385, df=1, p<0.001; Chi-square=5.466,
346 df=1, p≤0.019), while (2) survival was significantly higher in the first LPS exposure experiment (Log
347 Rank Mantel Cox; Chi-square=12.436, df=1, p<0.001), but not the second LPS exposure experiment
348 (Log Rank Mantel Cox; Chi-square=1.370, df=1, ns) in 0-6 hpf dexamethasone-treated larvae
349 compared to 0-6 hpf control-treated larvae.
350
351 *Phenotypical effects and gene expression endothelium-related genes*
352 In the first LPS exposure experiment phenotypical changes were measured at 4 and 28 hrs, i.e. 3.5
353 hrs and 27.5 hrs after ending exposure. Table 2 shows the numbers of dead larvae and of larvae that
354 were alive and displayed phenotypical changes. We observed LPS-induced changes in the shape of
355 the larvae (curved larvae), tail fin morphology (swollen or damaged tail fins) and heart cavity
356 (oedema). Larvae were scored as either affected (at least one of these changes present) or not (no
357 changes in any of the parameters). Supplementary table 1 shows the scores of the individual
358 parameters at 4 hrs (the number of larvae of the control treated group alive at 28 hrs was too low for a
359 meaningful statistical analysis between treatments).
360 While the scores at 4 hrs suggested that the LPS-induced effects were less strong in
361 glucocorticoid-treated larvae than in control-treated larvae, this was not (as yet) significant (Chi-
362 square=5.81, df=2, ns). LPS-induced effects were less strong in glucocorticoid-treated larvae than in
363 control-treated larvae at 28 hrs (overall Chi-square=25.33, df=2, p<0.001). Pair-wise comparison
364 showed that both cortisol-treated (Chi-square=18.05, df=2, p<0.001) and dexamethasone-treated
365 (Chi-square=18.34, df=2, p<0.001) groups showed fewer dead and fewer malformed larvae than
366 control-treated subjects following LPS exposure.
367 In addition to the phenotypical changes that we studied, we measured expression levels of
368 genes related to endothelial barrier function (*clnd5a*, *clnd2* and *oclnb*). Transcript abundance of *clnd5a*
369 was higher in cortisol-treated (Tukey HSD: p<0.001) and dexamethasone-treated larvae (Tukey HSD:
370 p≤0.067) compared to control-treated larvae (two-way ANOVA; treatment and time as independent

factor; treatment: $F(2,36)=9.815$, $p<0.001$; figure 5A). Transcript abundance of *clnd2* was lower in cortisol-treated (Tukey HSD: $p\leq0.034$) and dexamethasone-treated larvae (Tukey HSD: $p\leq0.093$) compared to control-treated larvae (two-way ANOVA; treatment and time as independent factor; treatment: $F(2,36)=9.815$, $p<0.001$; figure 5B). At 3 hrs transcript abundance of *clnd2* was higher than at other time points in all treatment groups (Tukey HSD; $p<0.001$; time: $F(2,36)=13.425$, $p<0.001$). Transcript abundance of *oclnb* was lower in cortisol-treated and dexamethasone-treated larvae than in control-treated larvae at baseline (0 hr), but higher at 3 hrs following LPS exposure (two-way ANOVA; treatment and time as independent factor; treatment *time: $F(3,36)=6.404$, $p<0.001$; figure 5C).

In the second LPS exposure experiment we measured changes in tail fin morphology and shape as we hardly observed any oedema in the heart cavity in the first LPS exposure experiment. Figure 6 shows the changes in tail fin morphology following LPS exposure. In control-treated larvae there was a clear and rapid loss of normal tail fin structure and a shift towards swollen or damaged tail fins, while this was not the case in the glucocorticoid-treated larvae. Statistical analysis showed that at 3 hrs (Chi-square=10.25, df=4, $p\leq0.036$), 6 hrs (Chi-square=10.27, df=4, $p\leq0.036$) and 24 hrs (Chi-square=10.25, df=4, $p\leq0.042$) treatment groups differed significantly from one another. More in particular, both cortisol-treated larvae (3 hrs: Chi-square=5.16, df=2, $p\leq0.076$; 6 hrs: Chi-square=6.94, df=2, $p\leq0.03$; 24 hrs Chi-square=5.77, df=2, $p\leq0.056$) and dexamethasone-treated larvae (6 hrs: Chi-square=6.98, df=2, $p\leq0.03$; 24 hrs Chi-square=6.53, df=2, $p\leq0.038$) showed less severe changes in tail fin morphology than control-treated larvae. We did not observe a strong effect of LPS exposure on the shape of the larvae. After 24 hrs only small percentages of each treatment group showed a curved shape: control-treated larvae (see supplementary table 2 for all time points): 15.8% (n=19); cortisol-treated larvae: 8.3% (n=24); dexamethasone-treated larvae: 9.1% (n=22); these differences were not significant (Chi-square=1.71, df=2, ns).

Gene expression analysis

First, we explored the effects of glucocorticoid treatment through the interrelationships of transcript abundance of genes using PCA. Then in each component, we selected genes of interest of which the transcript abundance of the different treatments matched the differences in phenotypical changes and survival that we observed between treatment groups.

A PCA revealed three components explaining in total 72.9% of variance (supplementary table 3). The KMO was sufficiently high (0.619) and Bartlett's test of sphericity was highly significant (Chi-square=303.010, df=55, $p<0.001$) indicating that the data were adequate for a PCA.

The first component, explaining 32.9% of the variance, consisted of the genes of different Toll-like receptors: *tlr2* (factor loading: 0.677), *tlr4ba* (0.669), *tlr4bb* (0.764), *tlr5a* (0.779) and *tlr5b* (0.838). The overall pattern of the factor regression scores across time was an inverted U-shape: a two-way ANOVA (independent factors: time and treatment) for this component revealed a highly significant effect of time ($F(3,36)=15.502$, $p<0.001$) with time points 0 hr and 3 hrs having significantly higher factor regression scores than time points 0.5 hr and 1 hr (Tukey HSD; supplementary Table 3). Only weak effects were found between the different treatments ($F(2,36)=3.012$, $p\leq0.062$; $F(6,36)=2.038$, $p\leq0.086$). The only gene of this component of which the transcript abundance of the different treatments seemed to match the differences in phenotypical changes and survival was *tlr4bb* (see supplementary table 4 and figure 7A): transcript abundance increased less strongly over time in glucocorticoid-medium treated subjects than in control-medium treated subjects.

The second component, explaining 23.1% of the variance, consisted of *il1β* (factor loading: 0.940), *myd88* (0.844) and *il10* (0.758). As time progressed factor regression scores increased (two-way ANOVA (independent factors: treatment and time); time: $F(3,36)=68.309$, $p<0.001$) with a post-hoc Tukey HSD revealing that the factor regression scores differed significantly from one another at all time points (supplementary table 3). The 0-6 hpf control group had increasingly higher factor regression scores than the 0-6 hpf cortisol treated group and the 0-6 hpf dexamethasone treated group as time progressed (supplementary table 3; Tukey HSD; treatment: $F(2,63)=22.664$, $p<0.001$; treatment*time: $F(6,36)=13.491$, $p<0.001$). Of all genes of this component, transcript abundance of the different treatments seemed to match the differences in phenotypical changes and survival (supplementary table 4 and figure 7 B-D): transcript abundance increased less strongly over time in glucocorticoid-medium treated subjects than in control-medium treated subjects.

The third component, explaining 16.9% of the variance, consisted of regulators of the immune response: *cxcr4a* (factor loading: 0.794), *cxcr4b* (0.809) and *ptpn6* (0.799). Overall the factor regression scores decreased over time: a two-way ANOVA (independent factors: treatment and time) revealed a highly significant effect of time ($F(3,36)=4.464$, $p\leq0.009$) with 3 hrs and 0 hr being significantly different from one another (Tukey HSD). The 0-6 hpf dexamethasone group had lower

factor regression scores than the 0-6 hpf control treated group and the 0-6 hpf cortisol treated group (treatment: $F(2,36)=11.056$, $p<0.001$; treatment*time: $F(6,36)=5.571$, $p<0.001$). The only gene of this component of which the transcript abundance of the different treatments seemed to match the differences in phenotypical changes and survival was *cxcr4a* (see supplementary table 4 and figure 7E): transcript abundance increased in control-medium treated subjects but decreased in glucocorticoid-medium treated subjects.

4. Discussion

The data of this study showed that treatment of zebrafish embryos with cortisol or dexamethasone during the first six hours after fertilization modulated the function of the immune system and thereby enhanced survival after an immune challenge. This suggests that in zebrafish maternal stress through enhancing oocyte cortisol levels and thereby increased GR stimulation leads to an adaptive response to immune challenges.

4.1. Baseline expression of immune-related genes

Hartig and colleagues (2016) have shown that 5-day exposure to (1 μM) cortisol in zebrafish embryos/larvae (0-5 dpf) enhanced baseline expression of immune-related genes such as of *socs3a*, *mpeg1* and *irg1l* at 5 dpf. Here, we show that exposure at the first six hours of life (0-6 hpf) is already sufficient to induce enhanced baseline expression of these genes at 5 dpf. As GR is the only corticosteroid receptor present in these early life stages (Alsop and Vijayan, 2008; Pikulkaew *et al.*, 2010, 2011), this suggests that activation of GR in these early stages is responsible for mediating these effects. Indeed, we show that 0-6 hpf exposure to the specific GR agonist dexamethasone enhanced baseline expression of these immune-related genes in 5 dpf larvae as well. This is in general agreement with data from studies showing that exposure of embryos/larvae to other GR agonists (for variable time-periods from fertilisation) increased baseline expression of (some of) these genes (Willi *et al.*, 2018, 2019; Zhao *et al.*, 2016).

We have previously observed that 0-6 hpf exposure to cortisol enhanced baseline levels of cortisol at 5 dpf in larvae of the AB strain but not in larvae of the TL strain (van den Bos *et al.*, 2019). In addition, we have observed that 0-6 hpf exposure to dexamethasone had no effect on baseline levels of cortisol at 5 dpf in larvae of the AB or TL strain (Althuizen, 2018). Overall these data suggest

460 that the effects on the expression of immune-related genes in AB and TL larvae are independent of
461 baseline levels of cortisol.
462 While we have previously observed substantial differences between larvae of the AB and TL
463 strains in gene expression, physiology and behaviour (van den Bos *et al.*, 2017a, 2017b, 2019, 2020),
464 the effects of 0-6 hpf exposure to cortisol and dexamethasone on baseline expression of immune-
465 related genes were similar in both strains. This suggests a robust strain-independent effect. Future
466 studies should elucidate whether this effect also holds in other strains revealing thereby fundamental
467 aspects of how early-life levels of cortisol may affect offspring functioning and survival.
468
469 **4.2 Tail fin amputation assay**
470 It has been shown that tail fin amputation in 3 dpf larvae leads to a rapid recruitment of macrophages
471 (within 2 hpa) remaining at a plateau for at least 24 hours thereafter, while the number of neutrophils
472 reaches a peak 4 hpa declining thereafter (Chatzopoulou *et al.*, 2016). Here, we observed that
473 dexamethasone increased the number of neutrophils at 4 hpa, while it had no effect on the number of
474 macrophages at 4 hpa. These data suggest that pre-exposed subjects mount a stronger response to
475 injury, possibly enhancing protection against invading micro-organisms (Hall *et al.*, 2014).
476 Dexamethasone, but not cortisol, exposure was effective in increasing the recruitment of
477 neutrophils. Two explanations may be forwarded for this. First, this may be related to
478 dexamethasone's higher affinity for GR (Rupprecht *et al.*, 1993). In line with this we observed
479 enhanced expression of FK506 binding protein 5 (*fkbp5*), a marker for levels of GR stimulation (e.g.
480 Willi *et al.,* 2018, 2019) in dexamethasone-exposed embryos, but not cortisol-exposed embryos
481 (Althuizen, 2018; van den Bos *et al.*, 2019). Second, it has been shown neutrophil recruitment, but not
482 macrophage recruitment, is sensitive to acute treatment with GR agonists, including cortisol, which
483 decreases the number of neutrophils at 4 hpa (Chatzopoulou *et al.*, 2016; Hall *et al.*, 2014; Xie *et al.*,
484 2019). We have observed that baseline levels of cortisol are enhanced following cortisol but not
485 dexamethasone treatment (Althuizen, 2018; van den Bos *et al.*, 2019). Hence, in the case of cortisol
486 pre-exposure enhancement of neutrophil recruitment through GR stimulation (as indicated by the
487 effect of dexamethasone) may potentially be offset at 3 dpf by the higher baseline levels of cortisol. If
488 so, this suggests a fine-tuning of the response. Future studies should address these alternative
489 explanations.

490

491 **4.3 LPS challenge**

492 In line with results from other studies (Dios *et al.*, 2014; Hsu *et al*., 2018; Novoa *et al.*, 2009; Philip *et*
493 *al.*, 2017) LPS exposure produced phenotypical changes (tail fin oedema, swollen or damaged tail
494 fins, and curved animals), mortality and increased expression levels of immune-related genes. To
495 assess the molecular mechanisms underlying the effects of LPS exposure, we measured the
496 expression of *cldn5a*, *cldn2* and *oclnb*; genes of which the expression has been shown to be changed
497 following LPS exposure (Hsu *et al*., 2018; Philip *et al.*, 2017) and which are involved in endothelial
498 barrier function (Kása *et al.*, 2015; Odenwald and Turner, 2013; Shen *et al.*, 2011; Yoseph *et al.*,
499 2016). The strong increase in the expression of *cldn2* at 3 hours in control-treated subjects is in line
500 with data from other studies (Hsu *et al*., 2018; Philip *et al.*, 2017). Increased expression of *cldn2* is
501 associated with endothelial hyper-permeability due to increased pore-pathway activity possibly
502 mediated by increased expression of IL-13 (Kása *et al.*, 2015; Odenwald and Turner, 2013; Shen *et*
503 *al.*, 2011; Yoseph *et al.*, 2016). At variance with other studies (Hsu *et al*., 2018; Philip *et al.*, 2017) we
504 did not observe strongly decreased expressions of *cldn5a* and *oclnb*. Decreased expression levels of
505 *cldn5a* and *oclnb* are related to endothelial hyper-permeability due a lower sealing function of the
506 pore-pathway and a less functional leaky pathway respectively (Kása *et al.*, 2015; Odenwald and
507 Turner, 2013; Shen *et al.*, 2011; Yoseph *et al.*, 2016). One reason for this may be that we measured
508 gene expressions at 3 hours after the start of the LPS exposure, while in other studies this was
509 measured at substantially later time points, i.e. 6 and 8 hours (Hsu *et al*., 2018; Philip *et al.*, 2017).

510 Both cortisol and dexamethasone exposure at 0-6 hpf were associated with milder effects to
511 LPS exposure (in both experiments), as indicated by milder phenotypical changes (lower number of
512 larvae expressing tail fin oedema, damaged tail fins or curved animals), lower mortality and lower
513 gene expression of immune-related genes, such as *il1β*. As to the expression of endothelium-related
514 genes we noted that the expression of *cldn5a* was higher and expression of *cldn2* lower in cortisol-
515 and dexamethasone-treated subjects compared to control-treated subjects. This suggests a lower
516 permeability of the endothelium due to a lower pore-pathway activity (Kása *et al.*, 2015; Odenwald and
517 Turner, 2013; Shen *et al.*, 2011; Yoseph *et al.*, 2016) supporting the milder phenotypical effects
518 following LPS exposure. The expression of *oclnb* was strongly increased after three hours in 0-6 hpf
519 cortisol-treated and dexamethasone-treated subjects compared to control-treated subjects. This

520 suggests lower permeability due to more protective leaky pathway activity (Kása *et al.*, 2015;
521 Odenwald and Turner, 2013; Shen *et al.*, 2011; Yoseph *et al.*, 2016). It has been shown that 24 hours
522 following LPS challenge in zebrafish larvae the expression of *oclnb* is strongly up-regulated facilitating
523 tissue-repair (Hsu *et al*., 2018). Again, the data support the milder phenotypical effects that we see
524 following LPS exposure in cortisol-treated and dexamethasone-treated subjects compared to control-
525 treated subjects.

526 To explore the underlying mechanisms of glucocorticoid treatment we measured the
527 expression of a series of genes of interest and related their expression to the outcome of the
528 phenotypical changes and mortality. Five genes stood out in this respect, reflecting two receptors
529 (*tlr4bb* and *cxcr4a),* a factor involved in the transduction pathway of the expression of cytokines
530 (*myd88*) and two cytokines (*il1β* and *il10*).

531 While LPS exerts its effects through transduction mechanisms following binding to TLR4 in
532 mammals (Goulopoulou *et al.*, 2015; Kása *et al.*, 2015), in zebrafish this is not clear as yet: *tlr4ba* and
533 *tlr4bb* have been suggested to be paralogues rather than homologues and TLR4BA and TLR4BB
534 have thus far not been shown to be activated by LPS possibly by lack of a binding site for LPS
535 (Sepulcre *et al.*, 2009; Sullivan *et al.*, 2009). However TLR4BB has been shown to be involved in
536 inflammatory processes as *tlr4bb* transcript abundance is increased following tail fin amputation
537 (Chatzopoulou *et al.*, 2016). Here, we observed an increase in *tlr4bb* expression in 0-6 hpf control-
538 treated subjects over time, accompanied by a strong inflammatory response, which was less strong in
539 0-6 hpf cortisol-treated or dexamethasone-treated subjects, accompanied by a milder inflammatory
540 response. This suggests a role for TLR4BB in the LPS-induced response.

541 In humans CXCR4 has been implicated in recognition of LPS or being part of a 'LPS sensing
542 apparatus' in addition to TLR4 (Triantafilou *et al.*, 2001, 2008). Furthermore, LPS increases the
543 expression of *cxcr4* through an NF-κB signalling pathway associated with increased micro-vascular
544 leakage in the lungs (Konrad *et al.*, 2017) or increased colorectal tumor metastasis (Liu *et al.*, 2017).
545 Here, we observed an increase in *cxcr4a* expression in 0-6 hpf control-treated subjects over time,
546 which was absent in 0-6 hpf cortisol-treated or dexamethasone-treated subjects. This difference in
547 gene expression may be associated with differences in the extent of vascular leakage as suggested
548 by the differences in the expression of genes involved in the endothelial barrier and differences in tail
549 fin oedema between treatments as discussed above. In zebrafish *cxcr4a* is found in endothelial cells

550 (blood vessels), while *cxcr4b* is not (Wei Chong *et al.*, 2001), which may explain that we only observed
551 a phenotype-related effect for the expression profile of *cxcr4a*. Interestingly, CXCR4 has been
552 implicated in the development of tolerance to lethal doses of LPS in zebrafish larvae (Dios *et al.*, 2014;
553 Novoa *et al.*, 2009). Thus, this suggests that CXCR4 is involved in modulating the response to LPS.
554 Overall therefore our data warrant further studies into the role of TLR4BB and CXCR4 in LPS-
555 induced sepsis in zebrafish as well as into the effects of early life glucocorticoid stimulation hereon.
556 Earlier studies have shown that MYD88 knockout larvae show enhanced survival to LPS
557 challenge (Hsu *et al*., 2018) and no increase in *il1β* expression (van der Vaart *et al.*, 2013). Lethal, but
558 not sub-lethal, doses of LPS have been found to be associated with high expression levels of *il1β* and
559 *il10* in zebrafish larvae, indicative of a hyper-inflammatory response (Dios *et al.*, 2014). In line with
560 these findings we observed that the expression levels of *myd88*, *il1β* and *il10* were strongly increased
561 3 hours after LPS challenge associated with low survival in 0-6 hpf control-treated subjects, but lower
562 levels of expression of all three genes with higher survival in 0-6 hpf cortisol-treated and
563 dexamethasone-treated subjects. Myd88 is an adaptor protein critical to toll-like receptor signalling
564 (except for TLR3; Goulopoulou *et al.*, 2016) and IL1β receptor signalling (see Kanwal *et al.*, 2013; van
565 der Vaart *et al.*, 2013) and thereby cytokine expression. It should be noted that *myd88* expression was
566 already low in 0-6 hpf cortisol-treated and dexamethasone-treated subjects, suggesting lower
567 transduction pathway activity, possibly leading to a lower stimulation of inflammatory pathways. It is
568 clear that this deserves further study.
569 As indicated above following cortisol or dexamethasone treatment at 0-6 hpf we noted
570 increased base-line expression levels of *irg1l*, *socs3a*, *mpeg1.1* and *mpeg1.2* compared to control
571 treatment at 0-6 hpf. These increased base-line levels may aid in increased clearance of bacteria and
572 preventing excessive inflammation and hence aid in increasing survival (Benard *et al.*, 2015; Hall *et*
573 *al.*, 2014; Jo *et al*., 2005).
574 The data of LPS exposure in larvae observed here seem to match the data of LPS exposure
575 in adult zebrafish following 0-5 dpf exposure to cortisol: LPS exposure did not increase *il1β* expression
576 in different tissues measured (Hartig *et al.*, 2016). Unfortunately no survival was measured in the latter
577 study.
578 Overall these data suggest that the response to LPS of subjects pre-exposed to cortisol or
579 dexamethasone is less strong than the response of subjects of pre-exposed to control medium. How

580 differences in survival, phenotype and gene-expression levels are causally related remains to be
581 studied. As we have observed that 0-6 hpf exposure to dexamethasone had no effect on baseline
582 levels of cortisol at 5 dpf in larvae of the AB strain (Althuizen, 2018), the data suggest that these
583 effects are independent of baseline levels of cortisol.
584
585 **4.4 Limitations**
586 A clear limitation is that we only used one dose of cortisol and dexamethasone. For convenience we
587 used equimolar doses of cortisol and dexamethasone, which may have led to different levels of
588 stimulation of GR 0-6 hpf. Thus, in future studies different dose-ranges may be warranted, e.g. to
589 study whether higher concentrations of cortisol in the tail fin amputation assay have an effect on
590 neutrophil recruitment.
591 Regarding the tail fin amputation assay it has been shown that glucocorticoids may play a role
592 in the differentiation of macrophages into a pro-inflammatory (M1) phenotype (Xie *et al.*, 2019). So,
593 future studies should study in more detail the effects of early life exposure of glucocorticoids on the
594 inflammatory response and wound healing. Similarly, we used LPS to induce a hyper-inflammatory
595 response, i.e. sepsis (Hsu *et al.*, 2018; Philip *et al.*, 2017), as a model to study the effectiveness of our
596 early life treatments. To assess the ecological relevance of our findings and their more general nature
597 the effects of early-life exposure on larval exposure to different pathogens, such as of bacterial, viral or
598 fungal origin, should be studied (see e.g. Meijer and Spaink, 2011; van der Vaart *et al.*, 2013).
599 We observed a variable response to the LPS challenge in the two exposure series. This is not
600 uncommon as we also observed variable responses in other immune-related paradigms such as the
601 response to a dextran sodium sulphate (DSS) challenge (van den Bos *et al*., unpublished
602 observations). While as yet speculative differences in baseline levels of expression of *il1β* may be one
603 associated factor as we observed that higher baseline levels seem associated with milder responses
604 to LPS (van den Bos *et al.*, unpublished data). This is not unprecedented as this has also been
605 observed in mice: enhanced levels of IL1 are associated with a milder response to LPS (Alves-Rosa *et*
606 *al.*, 2002). It has been shown that following hatching *il1β* expression increases due to exposure to
607 microbes in the medium (Galindo-Villegas *et al.*, 2012). This tunes the activity of the innate immune
608 system of the zebrafish larvae and determines thereby their disease resistance. In the laboratory
609 however this may lead to variation between experimental series as the microbial load of the medium

610 may vary from experimental series to series. It is clear that this warrants further studies. It has been
611 suggested that tuning the innate immune system occurs through chromatin modifications (see e.g.
612 Foster *et al.*, 2007; Galindo-Vargas *et al.*, 2012; Netea *et al.*, 2016, 2017). Our future studies are
613 directed at understanding the variable responses in this context.

614 The immune system affects HPI-axis activity and *vice versa* (Wendelaar Bonga, 1997).
615 However, we did not address changes in HPI-axis activity as a consequence of our procedures (tail fin
616 amputation or LPS exposure) in this study as the primary aim was to study whether our pre-treatments
617 would affect immune function. Still changes in HPI-axis activity may be anticipated. For example, a
618 recent study showed in European sea bass (*Dicentrarchus labrax*) larvae 5 day post-hatching that at
619 120 hours following infection with the bacterium *Vibrio anguillarum*, when mortality was already high,
620 HPI-axis activity increased (Reyes-López *et al.*, 2018). This increased activity coincided with
621 increased expression of pro-inflammatory and anti-inflammatory genes. While the underlying
622 mechanism was not clear as yet, the data show that infections may impact HPI-axis activity. It has
623 been shown that early-life exposure to cortisol dampens the response of the HPI-axis to stressors in
624 zebrafish larvae (Nesan and Vijayan, 2016). Hence, future studies should address how early-life
625 exposure to cortisol affects the relationship between immune function and HPI-axis activity, and how
626 this relates to the increased survival that we have observed here.

627 We have used 0-6 hpf cortisol exposure by the medium as a model or proxy of increased
628 oocyte levels of cortisol due to chronic stress in mothers (van den Bos *et al.*, 2019), while others have
629 used micro-injection of cortisol in the yolk of single-cell embryos (Best *et al.*, 2017; Nesan and Vijayan,
630 2012, 2016). Chronic stress, whether due to excessive predation, food shortage, crowding or out-of-
631 range-temperatures, is associated with increased levels of cortisol (Wendelaar Bonga, 1997) and at
632 this level these procedures may be a valid approach of mimicking maternal stress. Still, these different
633 stressors may have additional neuro-endocrine and/or metabolic signatures that affect oocyte yolk-sac
634 contents and hence thereby development of embryos and larvae. Thus, future experiments should
635 compare the current results to data from chronically stressed mothers using different types of
636 stressors.

637

638 **4.5 Conclusion**

These data show that early-life exposure to cortisol, as a model or proxy of maternal stress, induces an adaptive response to immune challenges, which seems mediated via the glucocorticoid receptor. These data are of relevance for both ecological research (Sopinka *et al.*, 2017) and biomedical research (Stewart *et al.*, 2014) in understanding the effects of stressful conditions and exposure to endocrine disruptors on disease susceptibility and survival of offspring.

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6. Conflict of interest

The authors have no conflict of interest to report.

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8. Contributions

RvdB and MS conceived the project. SC, KT, JA and RW performed experiments. RvdB, SC, KT, JA and RW analysed data. JZ and SC performed qPCR analysis. RvdB, GF and MS wrote the manuscript.

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903 **10. Legends figures**

904

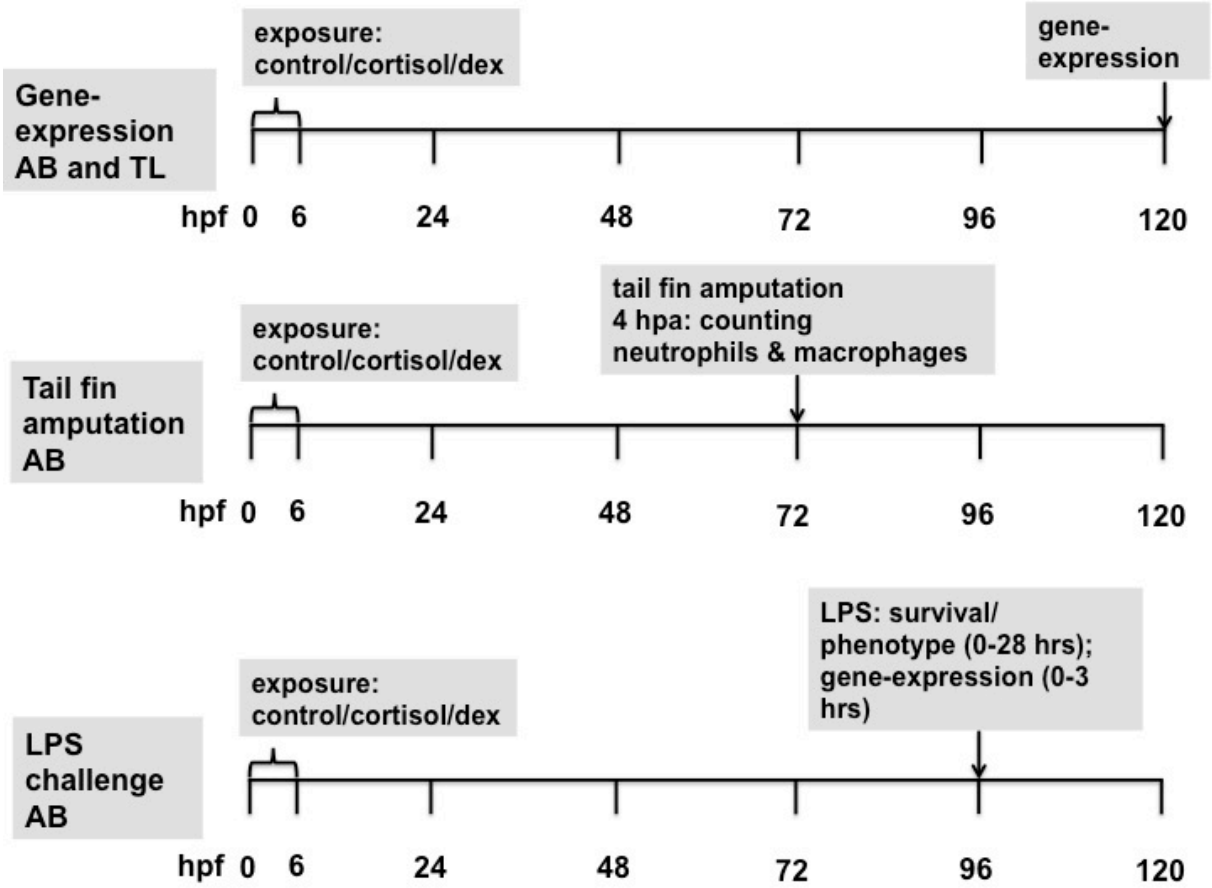


Figure 1: Experimental time-line of the different experiments.

dex=dexamethasone

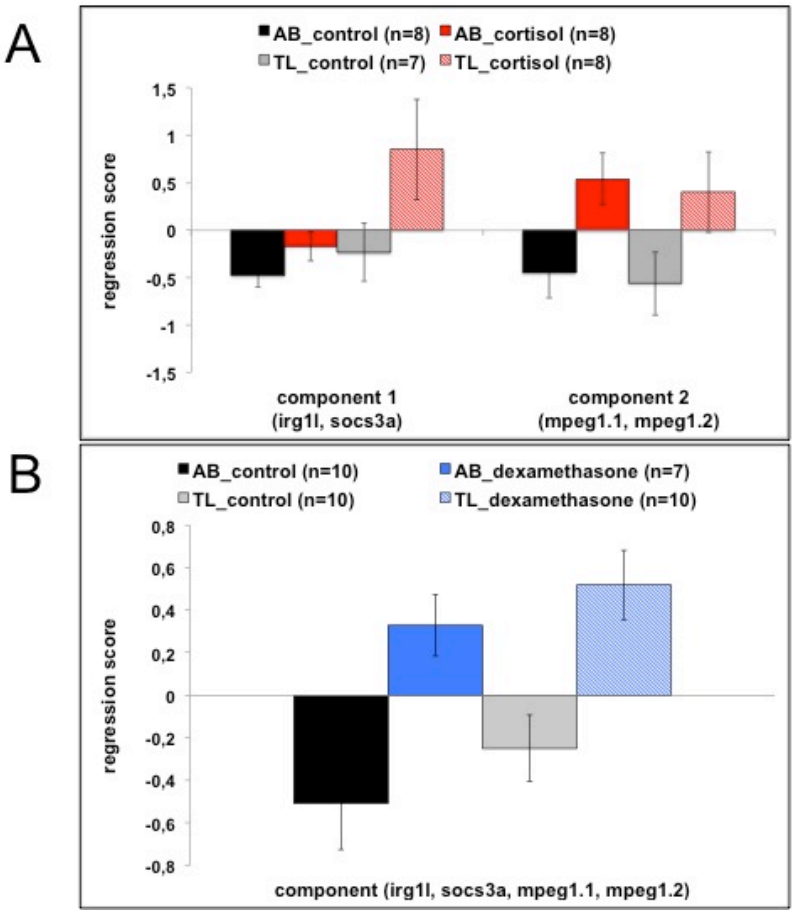


Figure 2, panel A: Regression scores (mean± SEM) of components 1 and 2 from the PCA for the different treatments (cortisol or control) and strains (AB or TL). Genes that contributed to the components are indicated in the figure. One subject was removed from the statistical analyses (TL control) as it was a consistent outlier following Grubb's outlier test.

Figure 2, panel B: Regression scores (mean± SEM) of the only component of the PCA for the different treatments (dexamethasone or control) and strains (AB or TL). Genes that contributed to the component are indicated in the figure.

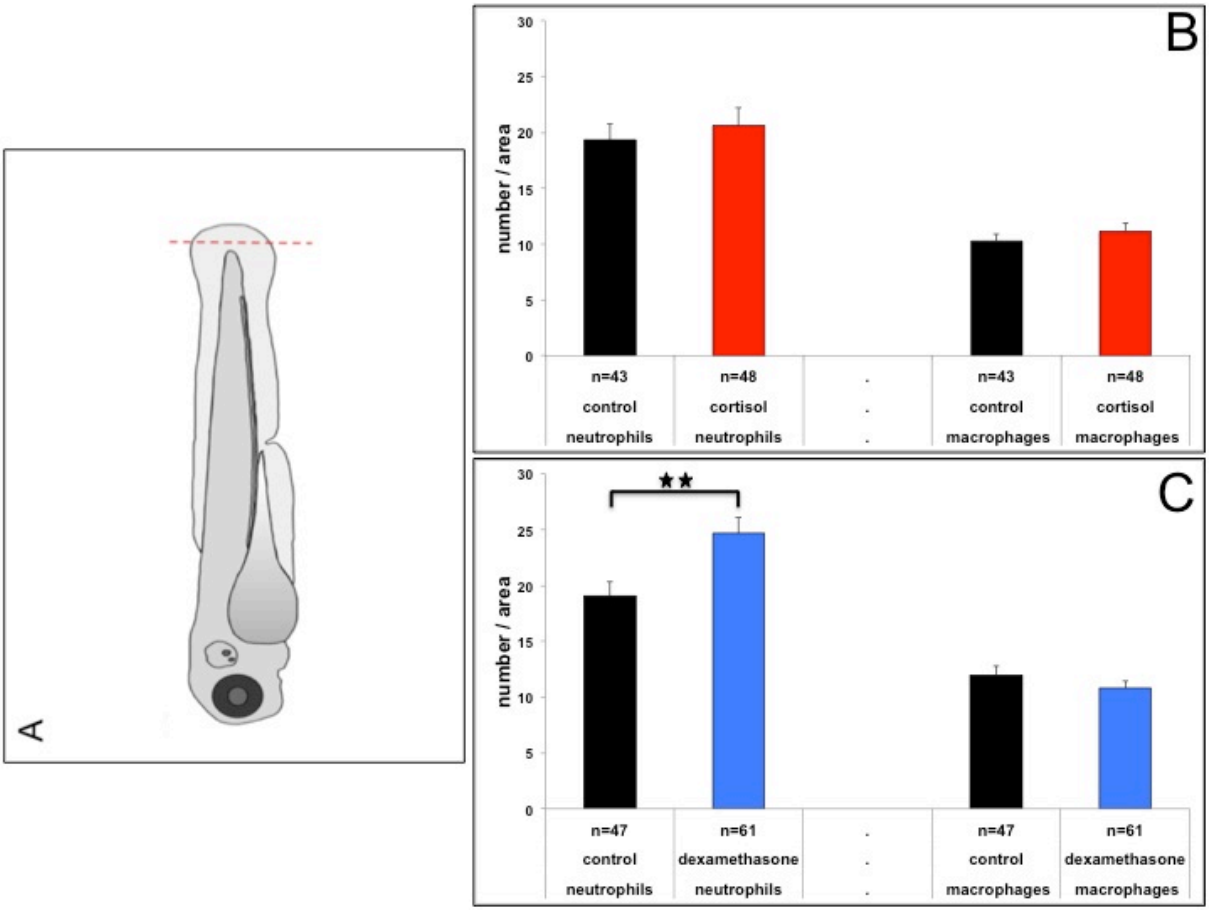


Figure 3, panel A Picture that illustrates the position of the site of the tail fin amputation in 3 dpf larvae.

Figure 3, panel B: Number of neutrophils and macrophages (mean± SEM) at 4 hpa for the different treatments (cortisol or control).

Figure 3, panel C: Number of neutrophils and macrophages (mean± SEM) at 4 hpa for the different treatments (dexamethasone or control).

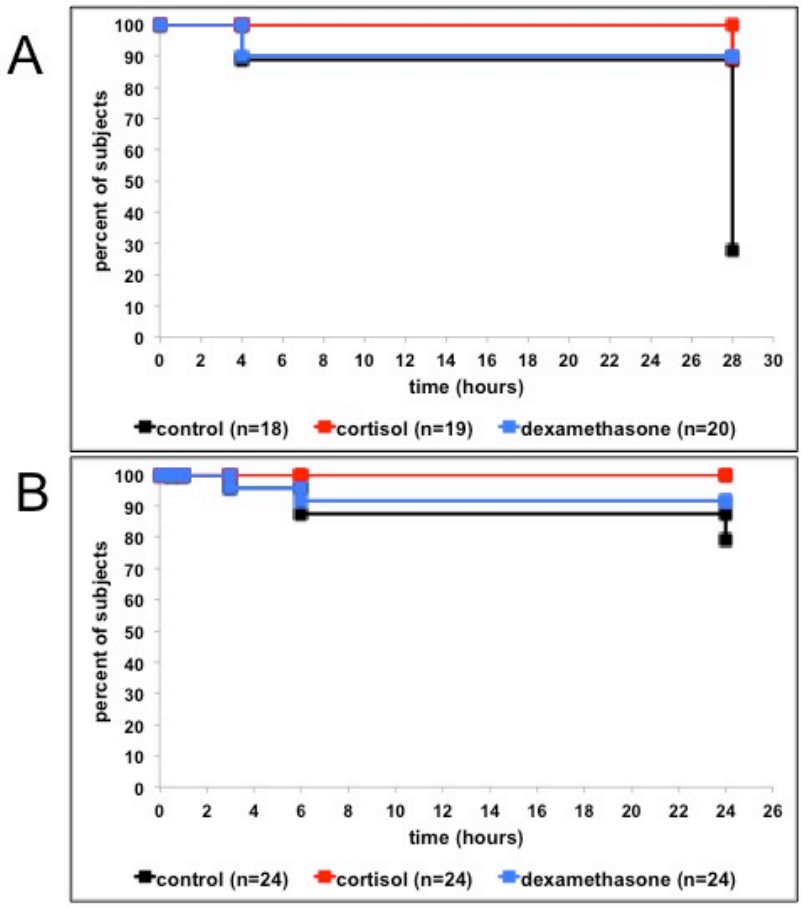
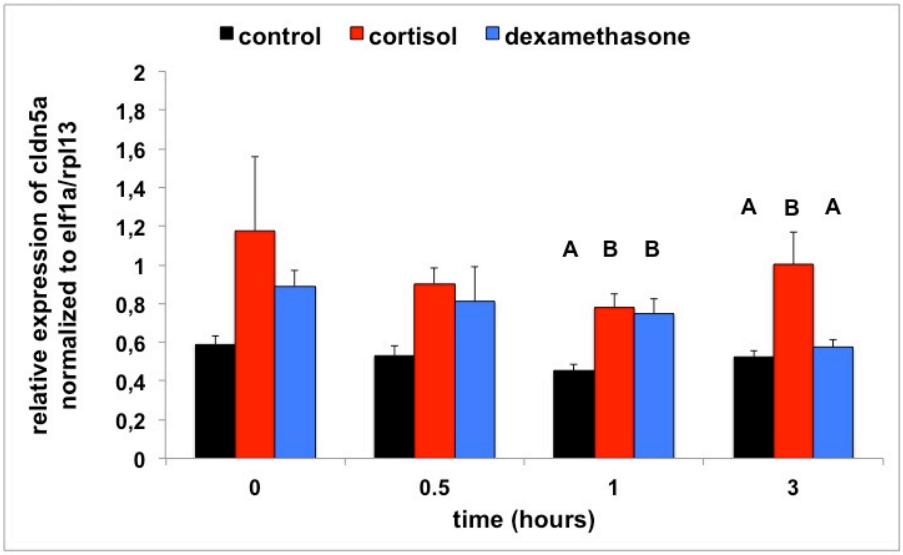


Figure 4, panel A: Per cent of surviving larvae following LPS treatment (from 0-0.5 hrs) and 0-6 hpf control treatment, cortisol treatment or dexamethasone treatment in the first exposure series (time-points: 0 hr, 4 hrs and 28 hrs).

Figure 4, panel B: Per cent of surviving larvae following LPS treatment (from 0-0.5 hrs) and 0-6 hpf control treatment, cortisol treatment and dexamethasone treatment in second exposure series (time-points: 0hr, 0.5 hr, 1 hr, 3 hrs, 6 hrs and 24 hrs).

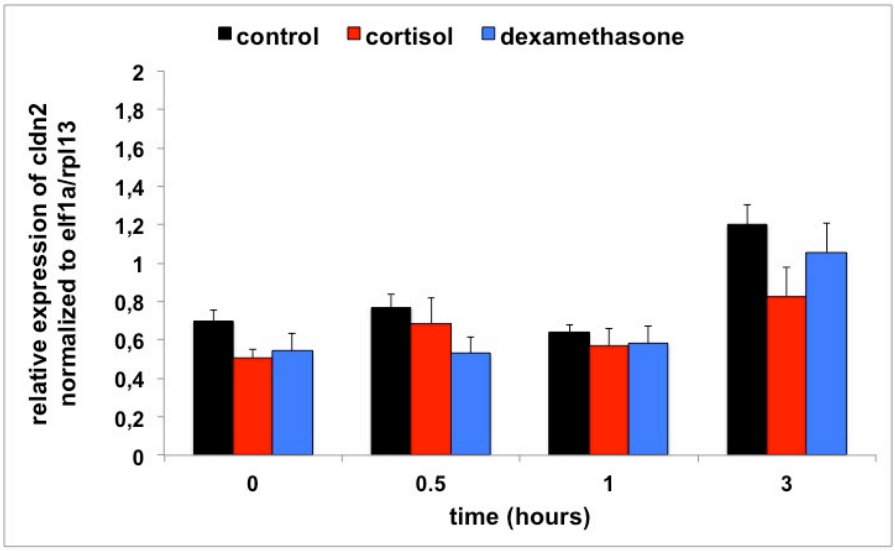
A



938

939 **Figure 5, panel A:** Transcript abundance (relative normalized expression; mean+SEM) of *cldn5a*
940 following LPS treatment (from 0-0.5 hr) and 0-6 hpf control treatment, cortisol treatment or
941 dexamethasone treatment (n=4 samples per time-point). Groups with the same capitals do not
942 significantly differ from one another (Tukey HSD following a significant treatment effect for this time-
943 point). Note: control-treated subjects showed no significant change over time: F(3,12)=2.009, ns.

B



944

945 **Figure 5, panel B:** Transcript abundance (relative normalized expression; mean+SEM) of *cldn2*
946 following LPS treatment (from 0-0.5 hr) and 0-6 hpf control treatment, cortisol treatment or
947 dexamethasone treatment (n=4 samples per time-point). Note: control-treated subjects showed an
948 increased expression at 3 hrs: F(3,12)=13.253, p<0.001; Tukey HSD.
949

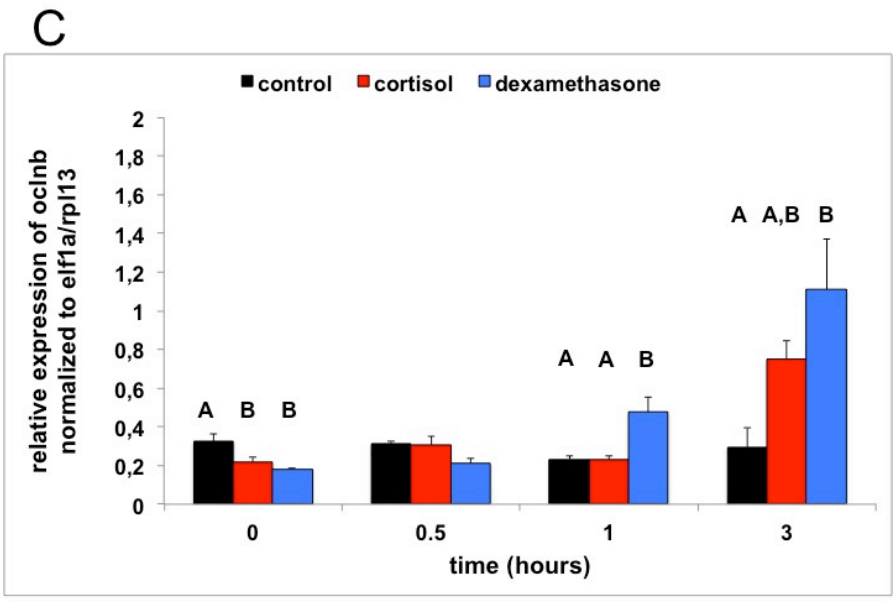


Figure 5, panel C: Transcript abundance (relative normalized expression; mean+SEM) of *oclnb* following LPS treatment (from 0-0.5 hr) and 0-6 hpf control treatment, cortisol treatment or dexamethasone treatment (n=4 samples per time-point). Groups with the same capitals do not significantly differ from one another (Tukey HSD following a significant treatment effect for this time-point). Note: control-treated subjects showed no significant change over time: $F(3,12)=0.562$, ns.

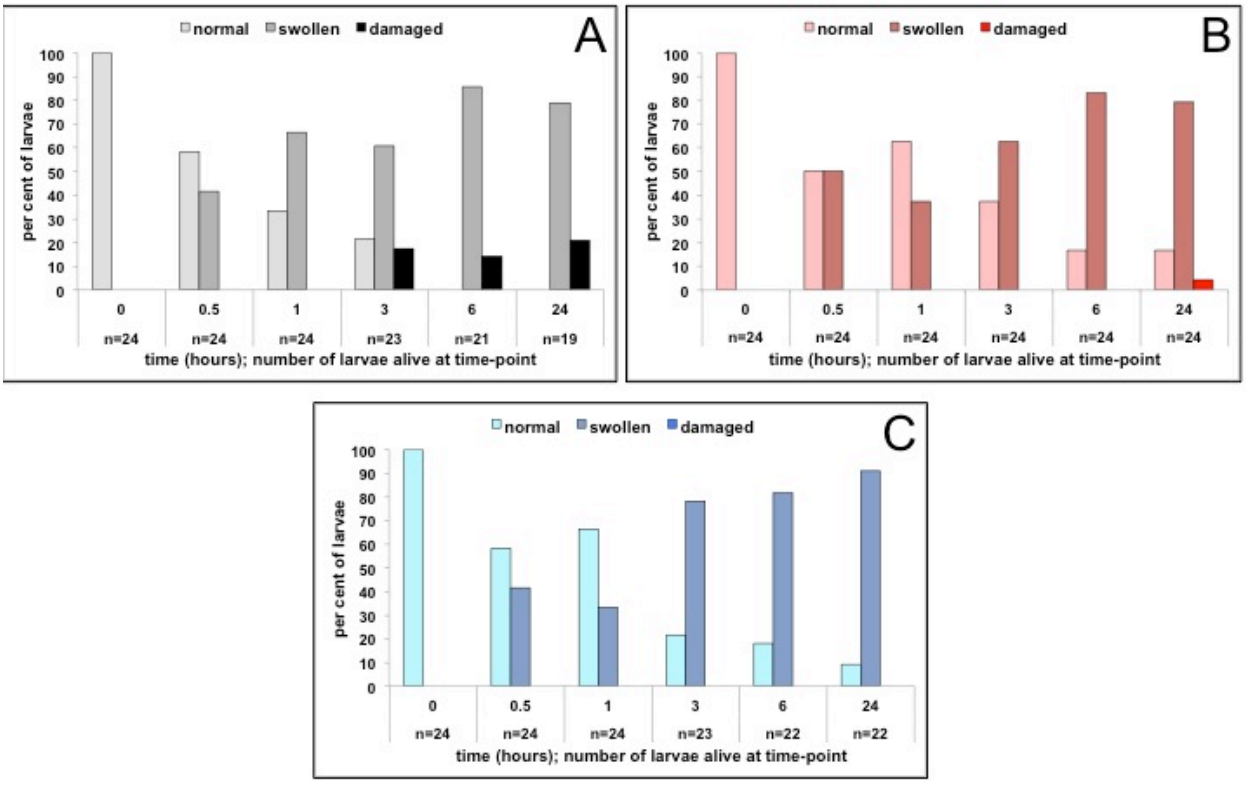
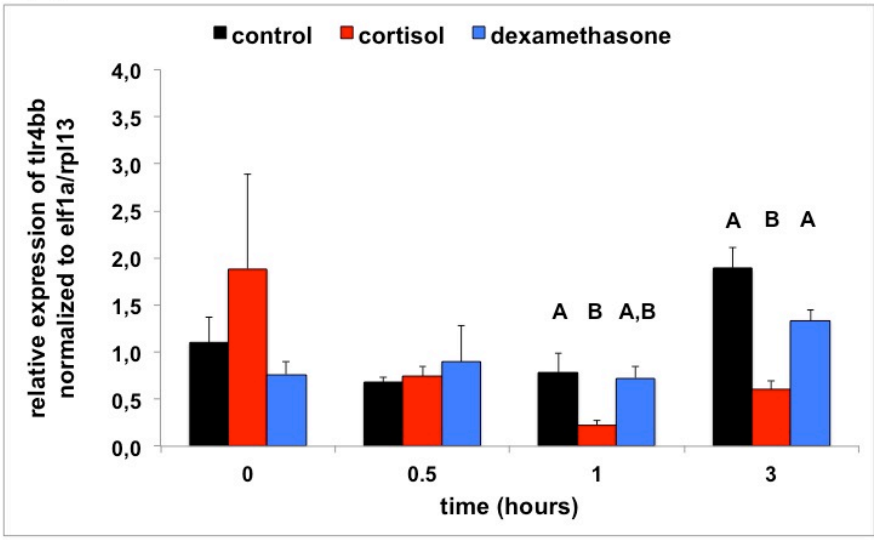


Figure 6, panel A: Per cent of larvae following LPS treatment (from 0-0.5 hr) in the 0-6 hpf control treatment group showing normal, swollen or damaged tail fins (time-points: 0hr, 0.5hr, 1 hr, 3 hrs, 6 hrs and 24 hrs).

Figure 6, panel B: Per cent of larvae following LPS treatment (from 0-0.5 hr) in the 0-6 hpf cortisol treatment group showing normal, swollen or damaged tail fins (time-points: 0 hr, 0.5 hr, 1 hr, 3 hrs, 6 hrs and 24 hrs).

Figure 6, panel C: Per cent of larvae following LPS treatment (from 0-0.5 hr) in the 0-6 hpf dexamethasone treatment group showing normal, swollen or damaged tail fins (time-points: 0 hr, 0.5 hr, 1 hr, 3 hrs, 6 hrs and 24 hrs).

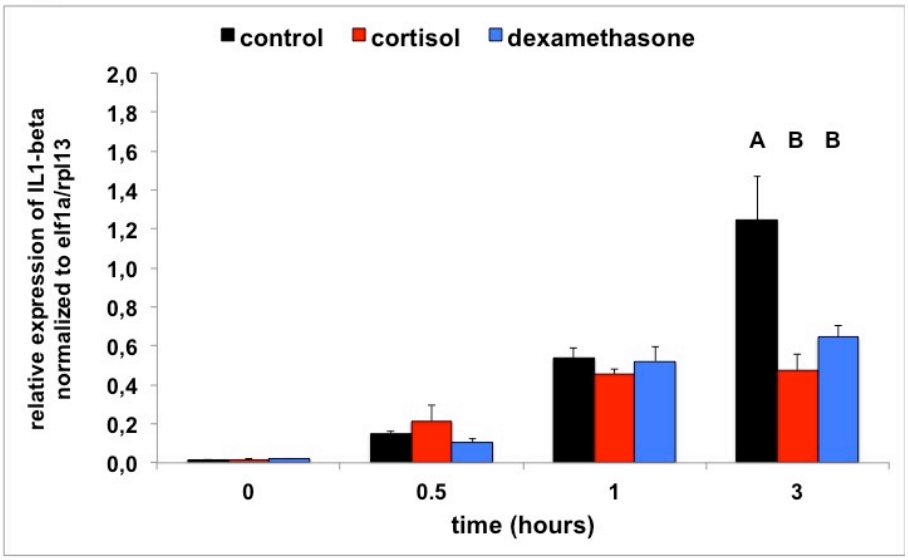
A



971

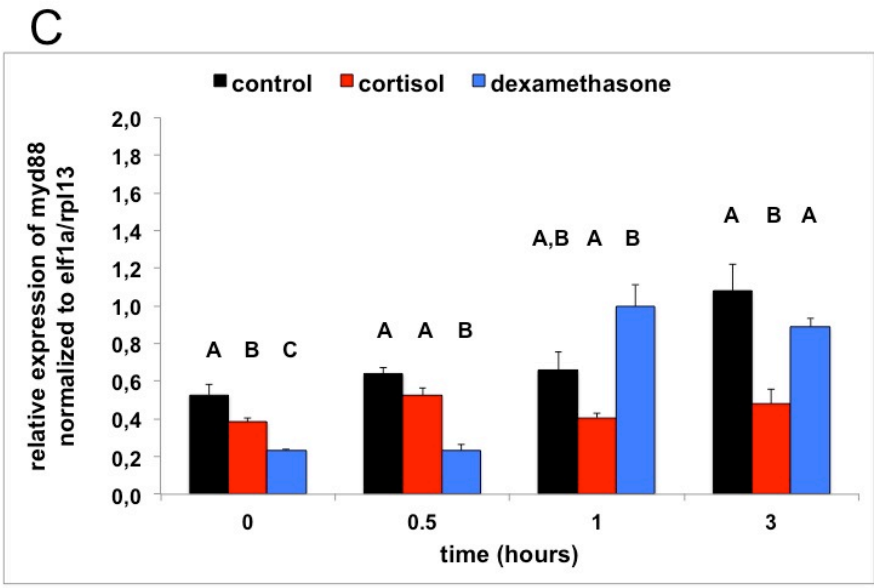
972 **Figure 7, panel A**: Transcript abundance (relative normalized expression; mean+SEM) of *tlr44bb*
973 following LPS treatment (from 0-0.5 hr) and 0-6 hpf control treatment, cortisol treatment or
974 dexamethasone treatment (n=4 samples per time-point). Groups with the same capitals do not
975 significantly differ from one another (Tukey HSD following a significant treatment effect for this time-
976 point).

B



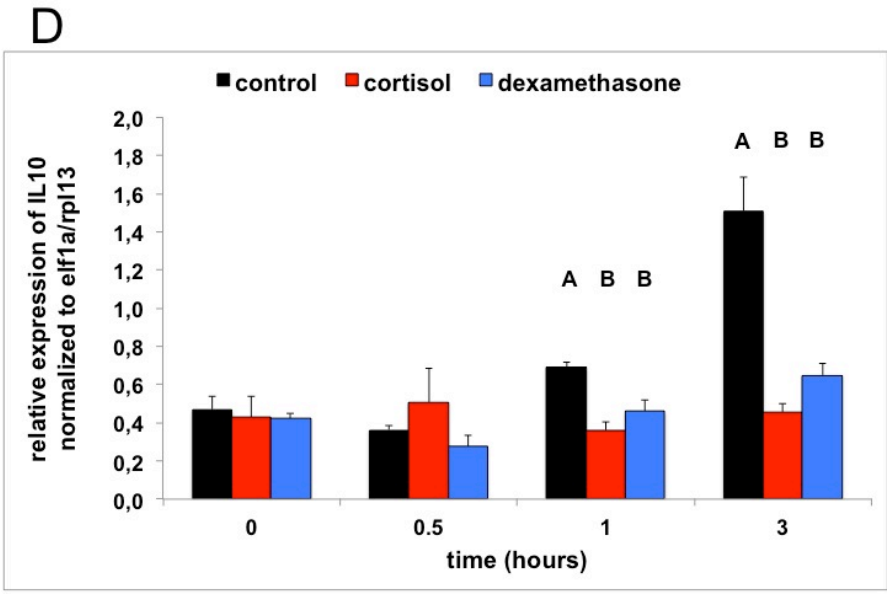
977

978 **Figure 7, panel B**: Transcript abundance (relative normalized expression; mean+SEM) of *IL1-beta*
979 following LPS treatment (from 0-0.5 hr) and 0-6 hpf control treatment, cortisol treatment or
980 dexamethasone treatment (n=4 samples per time-point). Groups with the same capitals do not
981 significantly differ from one another (Tukey HSD following a significant treatment effect for this time-
982 point).



983

984 **Figure 7, panel C**: Transcript abundance (relative normalized expression; mean+SEM) of *myd88*
985 following LPS treatment (from 0-0.5 hr) and 0-6 hpf control treatment, cortisol treatment or
986 dexamethasone treatment (n=4 samples per time-point). Groups with the same capitals do not
987 significantly differ from one another (Tukey HSD following a significant treatment effect for this time-
988 point).



989

990 **Figure 7, panel D**: Transcript abundance (relative normalized expression; mean+SEM) of *IL10*
991 following LPS treatment (from 0-0.5 hr) and 0-6 hpf control treatment, cortisol treatment or
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993 significantly differ from one another (Tukey HSD following a significant treatment effect for this time-
994 point).

E

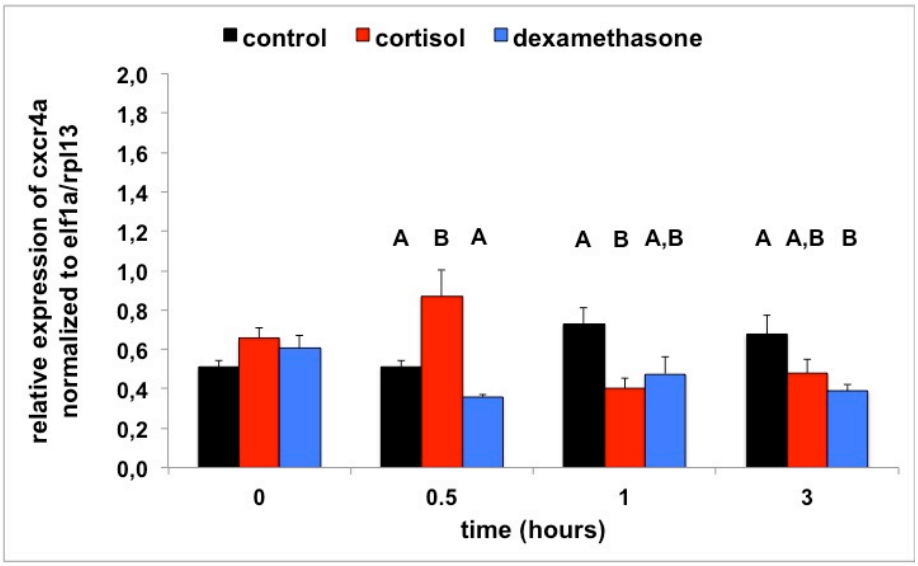
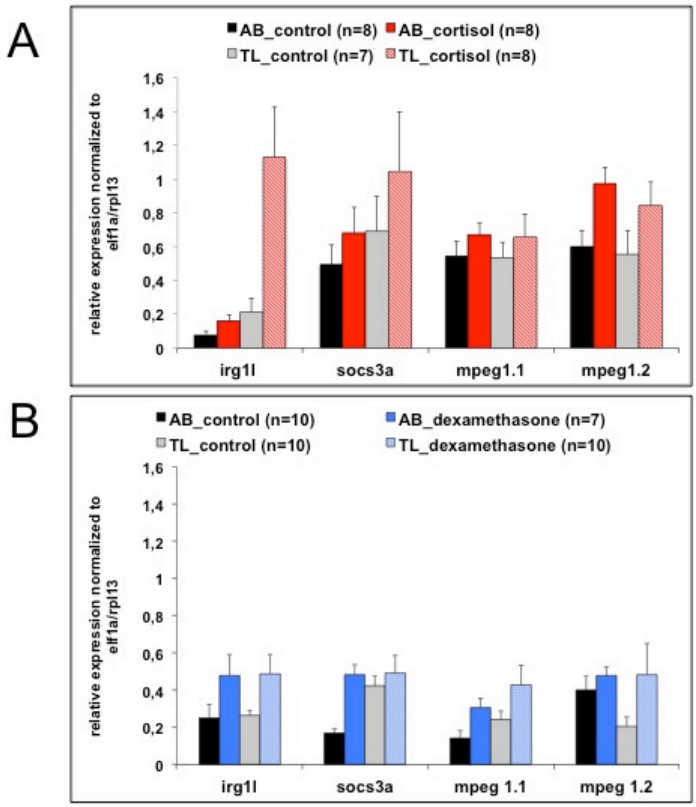


Figure 7, panel E: Transcript abundance (relative normalized expression; mean+SEM) of *cxcr4a* following LPS treatment (from 0-0.5 hr) and 0-6 hpf control treatment, cortisol treatment or dexamethasone treatment (n=4 samples per time-point). Groups with the same capitals do not significantly differ from one another (Tukey HSD following a significant treatment effect for this time-point).



Supplementary figure 1, panel A: Transcript abundance (relative normalized expression; mean+SEM) of the different immune related genes for the different treatments (cortisol or control) and strains (AB or TL). One subject was removed from the statistical analyses (TL control) as it was a consistent outlier following Grubb's outlier test. Treatment effects were found for *irg1l* ($F(1,23)=11.789$, $p \leq 0.01$; treatment * strain: $F(1,23)=8.446$, $p \leq 0.01$; AB: $p \leq 0.05$; TL: $p \leq 0.01$) and mpeg1.1 ($F(1,23)=8.614$, $p \leq 0.01$).

Supplementary figure 1, panel B: Transcript abundance (relative normalized expression; mean+SEM) of the different immune related genes for the different treatments (dexamethasone or control) and strains (AB or TL). Treatment effects were found for *irg1l* ($F(1,33)=6.484$, $p \leq 0.05$), *socs3a* ($F(1,33)=7.655$, $p \leq 0.01$) and mpeg1.1 ($F(1,33)=5.487$, $p \leq 0.05$).

Gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Amplicon length (bp)	Source
elf1a	CTGGAGGCCAGCTCAAACAT	TCAAGAAGAGTAGTACCGCTAGCATTAC	85	NM_131263.1
rpl13	TCTGGAGGACTGTAAGAGGTATGC	AGACGCACAATCTTGAGAGCAG	147	NM_212784
cldn5a	CTCGGGGGAAGCATACTCTG	TATTCCCCGTTGGATGTGGC	101	NM_213274.1
cldn2	CCCCTACTGGGCAACATCTG	CTTGCAGGTCGACGGTTAGT	155	XM_005170272.4
oclnb	TGACAAAGTCAGTGCTGCTCA	GGATATTCATCTGTGTCAAGCTCAT	162	NM_001008618.1
tlr2	TGCTGTCGGTCGATTACCTG	ACACAGGGAAAACGAAGGCT	130	NM_212812.1
tlr4ba	ACTTTCAAGATTTCCGTTGAGGTAT	CCACAAGAACAAGCCTTTGCAG	209	NM_001131051.1
tlr4bb	ATCACACTGCTGTGTTGCGA	AGGCCAGTGAGAGCTTTGTG	178	NM_212813.2
tlr5a	TGGTTCCGGTTGTGAGTTCC	TCTTGAAGATGACACCGCGT	162	XM_001919017.6
tlr5b	GGTCTTGAAGCGAGTGGTGT	CATTTTGCGCCAAGGTCAGT	118	NM_001130595.2
il1β	GATGGCATGCGGGCAATATG	AGCTCATTGCAAGCGGATCT	116	NM_212844.2
il10	GACCATTCTGCCAACAGCTC	ACCATATCCCGCTTGAGTTCC	102	NM_001020785.2
myd88	GTTTGCGCTCAGTCTTTGCC	GGTCAGAAAGCGCAGAATGC	103	NM_212814.2
cxcr4a	ACTTGTACAGCAGCGTCCTC	CTTTGGCGAACACCAAGTCG	174	NM_131882.3
cxcr4b	GCGCCTTTTTGAGCACACTT	ATTGCTGACTGAGAGGTCGC	127	NM_131834.1
ptpn6	ACAGGAAGAACTGCGCTCAT	TGCAGCACAGCCAAGTAGAT	225	NM_199960.1

Table 1: Nucleotide sequences of forward and reverse primers used for qPCR. Reference genes: elongation factor 1α (*elf1α*); ribosomal protein L13 (*rpl13*)

4 hrs	treatment	%dead	%(alive+affected) (tail fin, shape, heart)	%(alive+intact)
	control (n=18)	11.1	83.3	5.6
	cortisol (n=19)	0.0	73.7	26.3
	dexamethasone (n=20)	10.0	60.0	30.0
28 hrs	**treatment**	**%dead**	**%(alive+affected) (tail fin, shape, heart)**	**%(alive+intact)**
	control (n=15)	86.7	13.3	0.0
	cortisol (n=16)	12.5	37.5	50.0
	dexamethasone (n=16)	12.5	31.3	56.3

Table 2: Per cent of larvae dead, alive and affected (regardless of phenotypical changes in the tail fin, shape or cardiac area), and alive and intact at 4hrs and 28hrs after LPS treatment (0-0.5hr) in the different treatment groups: 0-6 hpf treatment with control, cortisol-containing or dexamethasone-containing medium. Note that the number of larvae at 28hrs is lower than at 4hrs; per treatment n=3-4 larvae (alive) were randomly selected for staining for reactive oxygen species in the tail fin (not reported here); they were not scored for phenotypical changes.