Detection of environmental glucocorticoids and investigation of their effects using the zebrafish embryo as a model

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Résumé

Les glucocorticoïdes synthétiques (GCs) sont largement utilisés comme des médicaments anti-inflammatoires. Ils imitent le cortisol, l'hormone naturelle du stress chez l'homme et chez le poisson. Le cortisol se lie et active le récepteur des glucocorticoïdes (GR) qui régule des gènes responsables du développement, de la réponse immunitaire, de la régulation osmotique et du comportement. Donc, la présence de GCs dans l'environnement aquatique peut provoquer une perturbation endocrinienne par l'altération des processus physiologiques essentiels chez le poisson. L'objectif de cette thèse est d'évaluer les effets des GCs à des concentrations relevantes pour l'environnement. L'intérêt principal est la réponse inflammatoire et les mécanismes moléculaires sur différents niveaux : le niveau des ARNm, des protéines et des métabolites.

Pour confirmer la présence de GCs dans l'environnement, l'activité des GCs a été déterminée le long d'une rivière suisse influencée par une station d'épuration des eaux en utilisant l'analyse d'effet orienté. Nous avons montré avec le test GR-CALUX® que les échantillons des effluents ont le plus grand potentiel d'activation du GR. L'analyse chimique a montré que le clobetasol propionate (CP), un GC extrêmement puissant, était responsable de 63 % de l'activité de GR totale.

Le CP a été choisi comme un produit chimique modèle pour étudier des effets du GC dans le poisson. Des embryons de poisson-zèbre (*Danio rerio*) jusqu'à 5 jours post fertilisation (dpf) ont été utilisés comme model in-vivo ; e travail avec les phases embryonnaires est une alternative plus douce à l'expérimentation animale. Nous avons démontré que le CP est accumulé rapidement par les embryons.

Les lipopolysaccharides bactériens (LPS) induisent une des cascades inflammatoires principales. Comme observé dans les tests de LPS, la réponse inflammatoire des embryons est réduite sensiblement après l'exposition au CP ≥0. 1 nM. Nous avons montré une régulation des ARNm de plusieurs des gènes qui sont associés au mécanisme des LPS et aux effets des GC. Parmi eux, Annexine a1b (*anxa1b*) est sous-régulé significativement après l'exposition au CP ≥0. 05 nM. Une sensible régulation d'anxa1b par le médicament anti-inflammatoire et non-stéroïdien diclofénac (DCF) a suggéré que cette protéine est un marqueur biologique potentiel pour les immunosuppresseurs stéroïdien et non-stéroïdien.

Une technique sur la base de CL-SM/SM a été développée pour mesurer des protéines sélectionnées, puisque les protéines sont les molécules qui sont responsable des fonctions cellulaires. 12 des 40 protéines cibles liées à l'action GC ont été détectées dans les embryons contrôles digérés. Par la suite, ils ont été mesurés après l'exposition au GC, au DCF et à l'eau récoltée dans la rivière. Nous avons constaté que la protéine du muscle

Myhz2 est régulée seulement après l'exposition au DCF. Par contraste, la protéine IκBα, une protéine anti-inflammatoire, est régulée seulement par le GC, en concordance avec les résultats de ARNm.

Les analyses globales et visées du métabolome ont été exécutées dans les embryons de poisson-zèbre exposés au CP de 4 à 5 dpf avec la collaboration avec M. Lamoree (VU Amsterdam). Plusieurs des molécules découvertes ont montré une réponse significative au CP : lysine, nicotinamide, choline, hypoxanthine, tyrosine et tryptophane.

Pour conclure, cette thèse démontre que les GCs sont présents dans l'environnement aquatique et peuvent réprimer la réponse inflammatoire des poissons lors de concentrations relevantes pour l'environnement.

Mots clé : poisson téléostéen, perturbation endocrinienne, glucocorticoïdes synthétiques, échantillons environnementals, analyse d'effet orienté, réponse inflammatoire, expression de gènes, marqueur biologique

Summary

Synthetic glucocorticoids (GCs) are widely used anti-inflammatory drugs. They mimic cortisol, the natural stress hormone in humans and in fish. Cortisol binds and activates the glucocorticoid receptor (GR), which regulates genes governing development, immune response, osmoregulation, and behavior. Therefore, the presence of GCs in the aquatic environment may cause endocrine disruption through impairing these essential physiological processes in fish. The aim of this thesis was to assess the effects of environmentally relevant concentrations of GCs in teleost fish, focusing on the inflammatory response and molecular mechanisms of GC action on different levels, from mRNA to proteins to metabolites.

To confirm the environmental presence of GCs, GC activity was determined along a Swiss freshwater stream impacted by a wastewater treatment plant, using effect-directed analysis. The effluent samples showed the highest potency to activate the GR, as determined by the GR-CALUX® assay. The chemical analysis found clobetasol propionate (CP), a highly potent GC, to be responsible for 63% of the total GR activity in the sample.

CP was selected as a model chemical to study GC effects in fish. Embryos of zebrafish (*Danio rerio*) aged until 5 days post fertilization (dpf) were used as a model organism; working with embryonic stages is a refinement of animal experiments. We demonstrated that CP is readily taken up by the embryos.

Bacterial lipopolysaccharides (LPS) induce one of the main inflammatory cascades. As observed in LPS challenge assays, the inflammatory response of the embryos was significantly reduced after exposure to ≥0.1 nM CP. We showed that mRNA expression of several LPS mechanism- and GC action-related genes was regulated by CP. Among them, Annexin a1b (*anxa1b*) was significantly down-regulated after exposure to ≥0.05 nM CP. Sensitive regulation of *anxa1b* by the non-steroidal anti-inflammatory drug diclofenac (DCF) suggested that this gene product is a potential biomarker of steroidal and non-steroidal immunosuppressive effects.

An LC-MS/MS-based targeted proteomics technique was developed to measure proteins, as it is these molecules that actually carry out cellular functions. 12 out of 40 GC action-related protein targets were detectable in control embryo digests and were subsequently monitored after CP, DCF and grab water sample exposures. We found that the muscle protein Myhz2 was regulated only after DCF exposure, while IκBα, an anti-inflammatory protein, was regulated only by CP, in agreement with mRNA level data.

Global and targeted metabolomics analyses were performed in zebrafish embryos exposed to CP from 4 to 5 dpf, in collaboration with M. Lamoree (VU Amsterdam). Several of the

detected compounds showed significant response to CP: lysine, nicotinamide, choline, hypoxanthine, tyrosine and tryptophan.

To conclude, this thesis demonstrates that GCs are present in the aquatic environment and may suppress the inflammatory response of fish at environmentally relevant concentrations.

Keywords: teleost fish, endocrine disruption, synthetic glucocorticoid, environmental samples, effect-directed analysis, inflammatory response, gene expression, biomarker

List of abbreviations

abbreviation	meaning
BCF	bioconcentration factor
CP	clobetasol propionate
DCF	diclofenac
DEX	dexamethasone
DEX-EQ	dexamethasone equivalent
DMSO	dimethyl sulfoxide
dpa	days post amputation
dpf	days post fertilization
dph	days post hatch
EDA	effect-directed analysis
EDC	endocrine disrupting chemical
EDP	European Demonstration Programme
GC	synthetic glucocorticoid
GR	glucocorticoid receptor
GRE	glucocorticoid responsive element
HPA axis	hypothalamic-pituitary-adrenal axis
hpc	hours post challenge
hpf	hours post fertilization
HPI axis	hypothalamic-pituitary-interrenal axis
IS	internal standard
LC-MS	liquid chromatography coupled to mass spectrometry
LC-MS/MS	liquid chromatography coupled to tandem mass spectrometry
LOD	limit of detection
LOQ	limit of quantitation
LPS	bacterial lipopolysaccharide
NSAID	non-steroidal anti-inflammatory drug
PTP	proteotypic peptide
RBA	respiratory burst assay
REP	relative potency
ROS	reactive oxygen species
SRM	selected reaction monitoring
WWTP	wastewater treatment plant

Contents

Chapter 1:

General introduction

Modern life style and our daily activities heavily depend on the use of various types of chemicals. Among them are pharmaceuticals, which include mimics of natural hormones, such as glucocorticoids. This thesis dealt with the identification of such chemicals in freshwaters and a wastewater stream and with the actions of such chemicals on fish.

1.1 Synthetic glucocorticoids in the aquatic environment

Synthetic glucocorticoids (GCs) are frequently prescribed drugs with anti-inflammatory and immunosuppressive effects. GCs were developed to be structurally similar to cortisol, the natural stress hormone in humans and fish, and thus they are potential endocrine disrupting compounds mimicking an endogenous hormone. These compounds interfere with the stress response axis, i.e. the hypothalamus-pituitary-adrenal (HPA) and hypothalamus-pituitary-interrenal (HPI) axes in humans and in fish, respectively. **Fig. 1.1** shows the HPI axis in fish. Mimicking cortisol, GCs bind to and activate the glucocorticoid receptor (GR) and regulate the expression of genes involved in, for instance, development, glucose metabolism, behavior, osmoregulation and immune response. Due to the complex role of cortisol, GCs can interfere with any of the physiological processes listed above. Because they can enter the environment via wastewater streams, they may pose a risk to non-target organisms having similar drug targets.

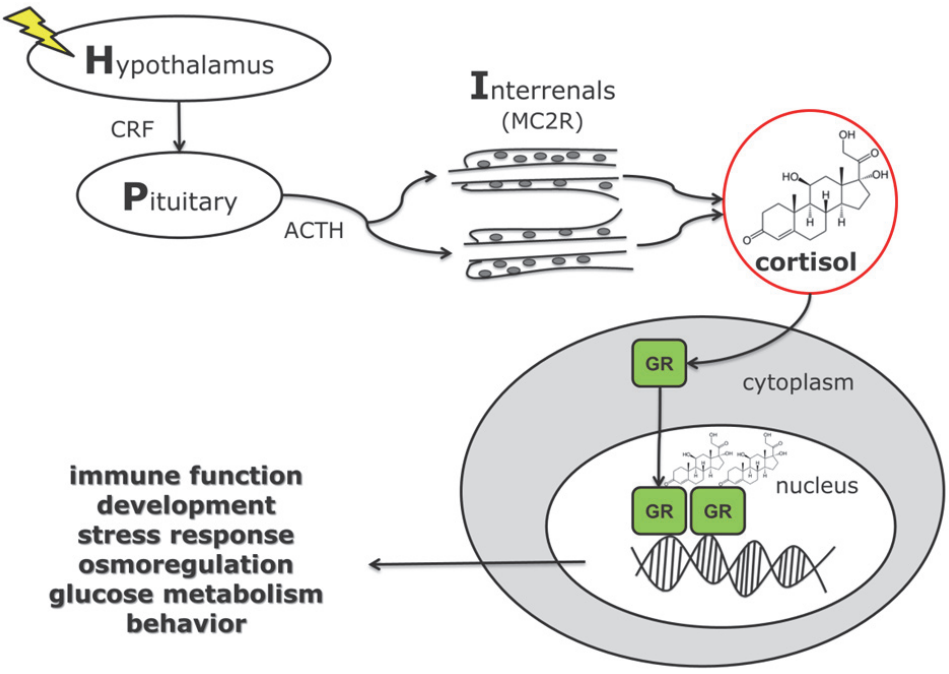

Figure 1.1: Hypothalamus-pituitary-interrenal (HPI) axis in fish. *Upon stress, the hypothalamus is activated, producing the corticotropin-releasing factor (CRF). CRF induces the pituitary synthesis of adrenocorticotropic hormone (ACTH), which is subsequently released into the blood stream. ACTH is recognized by the melanocortin 2 receptor (MC2R) in the interrenal tissue. In response, cortisol is synthesized and released. Cortisol binds to and activates the glucocorticoid receptor (GR), which is found in almost all cell types. Upon activation, GR translocates into the nucleus, and after homodimerization it either directly binds to glucocorticoid-responsive elements (GREs) or indirectly affects the gene expression through interactions with other transcription factors. GR regulates complex physiological phenomena, such as immune function, development, stress response, behavior and osmoregulation.*

The presence of GCs in the aquatic environment can be measured by chemical analysis targeting known GCs. GCs have been found in the aquatic environment all over the world (Kugathas et al., 2012). Kugathas et al. recommended using the concentration range from 10 to 1000 ng/L for risk assessment of these compounds, as their predicted total GC concentration in the river Thames was 30-850 ng/L. This concentration range was predicted based on average consumption of the population in the catchment and removal of the individual compounds in the waste water treatment plants (WWTPs). Since then, several studies quantified selected GCs in the aquatic environment by chemical analysis. The total detected GC concentrations were found to be up to 57 ng/L in surface waters, up to 100 ng/L in WWTP effluents and up to 836 ng/L in WWTP influents (Ammann et al., 2014; Herrero et al., 2014; Iglesias et al., 2014; Jia et al., 2016; Liu et al., 2015; Macikova et al., 2014; Nakayama et al., 2016).

The concentration of GCs in environmental samples can also be determined through their biological effect. GR-CALUX®, an *in vitro* reporter gene assay based on ligand-binding and activation of the GR, was developed to measure the total concentration of GR-active compounds in environmental samples (Van der Linden et al., 2008). Individual GCs can be characterized by their potency to activate the GR. The potency is normalized to the standard GC dexamethasone (DEX), providing relative potencies (REP). Thus, the total GR activity measured by the GR-CALUX® or similar bioassays is given in ng/L DEX equivalents (DEX-EQs). Studies using this approach have detected GR activity up to 2.7 ng/L DEX-EQs in surface waters and up to 155 ng/L DEX-EQs in WWTP effluents (Creusot et al., 2014; Jia et al., 2016; Macikova et al., 2014; Schriks et al., 2013; Stavreva et al., 2012; Suzuki et al., 2015; Van der Linden et al., 2008).

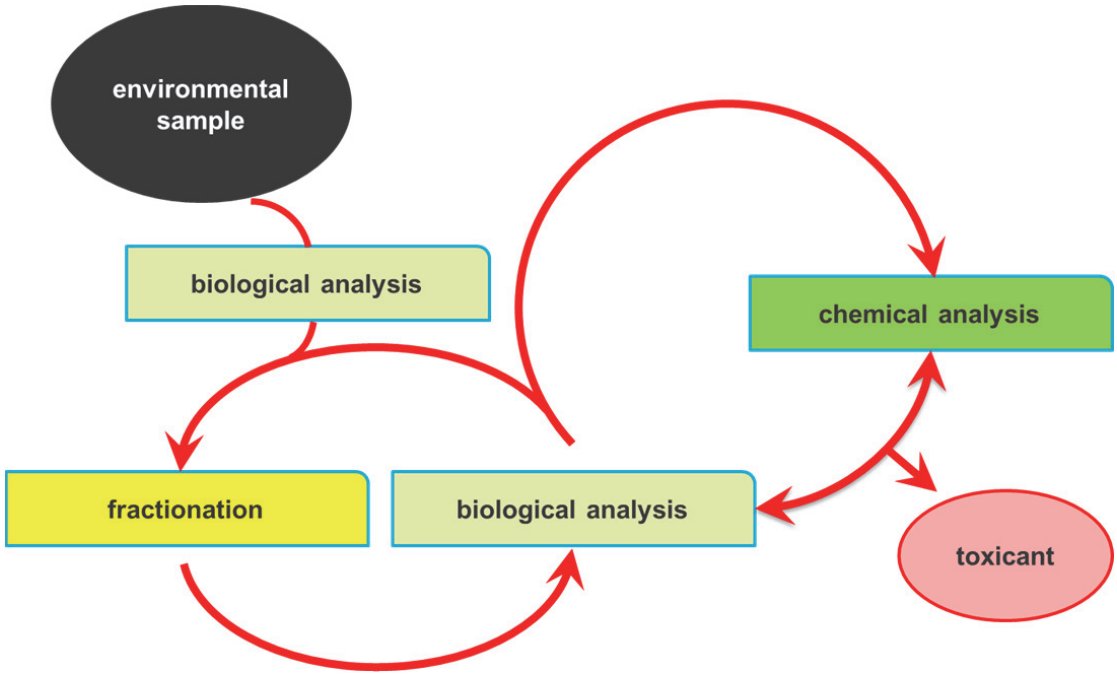

Figure 1.2: Effect-directed analysis (EDA) (Brack, 2003). *The environmental sample is first assessed by a mode-of-action specific bioassay, such as the GR-CALUX®. In case activity is detected, the sample is fractionated and the fractions are examined with the same bioassay in order to narrow down which fractions contain the active compounds. This process may be repeated in order to reduce the complexity of the fractions. The active fractions are then assessed by chemical target analysis to determine whether any pollutants known to cause the biological effect are present in the sample. If the biological activity cannot completely be explained by the detected chemicals, non-target chemical analysis has to be carried out to identify unknown biologically active compounds.*

The GC activity measured by bioassay analysis and predicted based on the target chemical analysis can be compared in order to determine the compounds responsible for biological effects in complex mixtures. This approach is the basis of effect-directed analysis (EDA), schematically shown in **Fig. 1.2** (Brack, 2003).

So far, there have been only two studies using EDA for glucocorticoids (Creusot et al., 2014; Macikova et al., 2014), and two others that combined chemical and biological assays to determine GC concentrations in GR-active environmental water samples without fractionation (Chen et al., 2016; Jia et al., 2016). In the study conducted by Jia et al, clobetasol propionate (CP), fluticasone propionate, triamcinolone acetonide and fluocinolone acetonide were found to be predominantly responsible for the detected biological activity in WWTP effluents. The authors found a good agreement between the biological activity and measured chemical concentrations. Chen and coworkers analyzed the GR activity with an *in vitro* assay and measured the three GCs DEX, triamcinolone and prednisolone in WWTP effluent samples. They could not explain all the biological activity with the detected

concentrations of these three GCs. They also exposed adult male zebrafish and embryos to the three GCs and the GR-active WWTP effluent. They determined that gene expression patterns after GC exposure were sufficiently robust to detect the same effects in the effluent-exposed zebrafish. Macikova and coworkers identified betamethasone/DEX, budesonide and flumethasone to be the main contributors to GR activity of surface water samples. Betamethasone/DEX, CP, and betamethasone/DEX 21-acetate were determined as the main contributors in WWTP effluents. They found that the biological activity calculated from chemical concentrations explained the biological activities, or in some cases was even higher, suggesting the presence of GR antagonistic compounds in the samples. Creusot et al found DEX and 6α-methylprednisolone as the contributors to GR activity. However, they could not explain all the biological activity with these compounds. This could be due to the fact that they did not monitor compounds that have been identified in the other studies as the main contributors to GR activity, such as CP.

1.2 Effects of GCs in fish

A number of studies have explored potential harmful effects of GCs in fish, though mostly at concentrations above those expected or detected in the environment. A selection of these studies is presented in **Table 1.1**. Most studies were performed with zebrafish (*Danio rerio*) and more specifically with zebrafish embryos. Zebrafish is an excellent model for GC research as the molecular components of the HPI axis are present by 2 days post fertilization (dpf), and functional by 3 to 4 dpf (Alderman and Bernier, 2009; Alsop and Vijayan, 2009; Nesan and Vijayan, 2016; Schaaf et al., 2009). The zebrafish genome is sequenced and extensively annotated, thus molecular biology tools are available to decipher molecular mechanisms. Furthermore, using the zebrafish embryo as a model until 5 dpf, i.e. during the so-called non-protected life stage, is considered an alternative to conventional animal testing (Strahle et al., 2012).

In summary, the studies listed in **Table 1.1** found that GC exposure impacted morphological development, impaired tissue regeneration after injury, affected osmoregulation, caused anxiety-like behavior, enhanced gluconeogenesis, and interfered with the immune response of fish. Studies that found effects after exposing fish to GC concentrations predicted or measured in the aquatic environment (marked by * in the concentration column of **Table 1.1**) found morphological alteration of the lower jaw, increased swimming and hatching rate after stimulus (McNeil et al., 2016), and effects on reactions mediated by the hepatic enzymes CYP450 (Burkina et al., 2015). Gluconeogenesis was induced as shown by the increased free amino acid serum levels (Nakayama et al., 2014). Furthermore, plasma glucose levels were elevated and the gluconeogenetic gene phosphoenolpyruvate carboxykinase (pepck) was induced (Carney Almroth et al., 2014; Chen et al., 2016; Kugathas et al., 2013;

Kugathas and Sumpter, 2011; Margiotta-Casaluci et al., 2016). The immune response was affected as well, as indicated by reduced white blood cell counts (Kugathas et al., 2013; Kugathas and Sumpter, 2011; Margiotta-Casaluci et al., 2016).

Table 1.1: Studies investigating the effects of GCs in fish after aqueous exposure

Fish species	GC	Concentration (µM)	Solvent vehicle	Exposure duration	Observed effects	Reference
zebrafish (*Danio rerio*)	BEC	0.25	DMSO	1-4 d, after 1 d cardiac injury	impaired cardiac repair, excessive collagen deposition, interfering with the inflammatory response in heart tissue: il-1β il-8, tnf-α, ptgs-2b, mpx expression reduced, lipocortin-1 (anxa1b) induced	Huang et al., 2013
zebrafish (*Danio rerio*) embryos	BEC	25	DMSO	3 dpf: pre-exposure before caudal fin amputation	transcriptomics → mainly immune-related genes regulated after amputation, that was mostly suppressed by GC exposure. However, for example anxa1a was induced.	Chatzopoulou et al., 2016
fathead minnow (*Pimephales promelas)*	BDP PRE	0.0019* 0.0028*	EtOH	21 d	increased plasma glucose, reduced leukocyte number	Kugathas and Sumpter, 2011
fathead minnow (*Pimephales promelas)*	BDP	0.00002, 0.0002, 0.002*	DMF	21 d	blood glucose elevated, liver PEPCK and GR expression increased, lymphocyte count decreased	Margiotta-Casaluci et al., 2016
fathead minnow (*Pimephales promelas)*	BDP	0.0002, 0.002, 0.02*	EtOH	21 d	≥ 0.0002 µM: increase in glucose levels, PEPCK and GR expression; ≥0.002 µM: Decrease in plasma CORT and blood lymphocyte count; ≥0.02 µM: decrease in plasma VTG	Kugathas et al., 2013
zebrafish (*Danio rerio*)	BEC	0.25	DMSO	1-4 d, after 1 d cardiac injury	impaired cardiac repair, excessive collagen deposition, interfering with the inflammatory response in heart tissue: il-1β il-8, tnf-α, ptgs-2b, mpx expression reduced, lipocortin-1 (anxa1b) induced	Huang et al., 2013
zebrafish (*Danio rerio*) embryos and adult	BDP, CORT, TRI, CLOB, HAL,	≤25	DMSO	2-5 dpf (3 dpa)	tissue regeneration inhibited	Mathew et al., 2007

	BDP	1 (BDP)	DMSO	adult (5 dpa), 2-3 dpf (1 dpa)	FKBP5, GILZ, sox9b up-regulated	Mathew et al., 2007
zebrafish (*Danio rerio*) embryos	BV	1	was used, not specified	2-4 dpf	POMC expression suppressed	Schoonheim et al., 2010
zebrafish (*Danio rerio*) embryos	CORT DEX	0.5 1	DMSO	2-4 dpf	increased Na^+ uptake	Kumai et al., 2012
coho salmon (*Oncorhynchus kisutch)*	CORT	2.8	EtOH	1 d	3 months old juvenile fish: increased dominance behavior and increased boldness	Sopinka et al., 2015
medaka (*Oryzias latipes*)	CORT	5		0-5 dph, females	suppressed proliferation of female type germ cells and the expression of cyp19a1 and induced gsdf	Kitano et al., 2012
zebrafish (*Danio rerio*) embryos	CORT	10	DMSO	2-4 dpf	epithelial permeability reduced, increased abundance of tight junction proteins occludin-a (no change on mRNA level) and claudin-b (up-regulation on the mRNA level too)	Kwong and Perry, 2013
zebrafish (*Danio rerio*) embryos	CORT	55.2	-	0-1 dpf 0-3 dpf	expression of GR down-, gcm2, nhe3b, atp6v1a, ecac up-regulated HRC, NaRC increased	Cruz et al., 2013
zebrafish (*Danio rerio*) embryos	CORT	55.2	-	0-3 dpf	increased Ca^{2+} influx, expression of ecac, hsd11b2, down-regulated 11β- hydroxylase and GR; increased acid secretion function and expression of transporters related to this mechanism.	Lin et al., 2015; Lin et al., 2011
zebrafish (*Danio rerio*) embryos	CORT DEX	275.9 254.8	DMF	3-24 hpf 3-48 hpf 3-72 hpf 3-96 hpf	Morphological changes including craniofacial deformities; induction of mmp-2,-9,-13	Hillegass et al., 2007; Hillegass et al., 2008

common carp (*Cyprinus carpio*)	CP CB	0.002*	DMSO	21 d	free amino acid serum concentrations increased	Nakayama et al., 2014
rainbow trout (*Oncorhynchus mykiss)*	DEX	0.000008, 0.00008, 0.0008, 0.008*	-	21 d 42 d	effect on CYP450-mediated reactions (EROD, BCFOD, BQOD, PNPH)	Burkina et al., 2015
zebrafish (*Danio rerio*) embryos and adult	DEX, PRE, TRI, GR-active secondary WWTP effluent	0.00005, 0.0005, 0.005, 0.05*	DMSO	3-123 hpf adult male: 4 d	pepck, baiap2, pxr up-regulated baiap2, pxr, mmp-2 up-regulated	Chen et al., 2016
fathead minnow (*Pimephales promelas)*	DEX	1.3	-	1) 21 d, adult female 2) 29 d embryo-larval	1) reduction in fecundity, plasma estradiol conc, increased occurrence of abnormally hatched fry 2) increase in deformed gill opercula	LaLone et al., 2012
fathead minnow (*Pimephales promelas)*	DEX	1.5	DMF	<48 h – 28 dph	decrease in survival	Overturf et al., 2012
zebrafish (*Danio rerio*) embryos	DEX-21 phosphate	5.1, 51, 510	-	4-5 dpf	lower body weight and anxiety-like behavior in adult males (assessed at 120 dpf)	Khor et al., 2013
Senegalese sole (*Solea senegalensis*)	DEX	25.5	EtOH	21 dph – for two weeks	reduced growth, increased susceptibility to pathogens, regulation of genes involved in the innate system, HPI and GH/IGF axes and cellular defense	Salas-Leiton et al., 2012
zebrafish (*Danio rerio*) embryos	DEX (water soluble)	40	-	1-2 dpf 1-3 dpf, 1-5 dpf	POMC expression suppressed	To et al., 2007

zebrafish (*Danio rerio*) embryos	DEX	100	not mentioned	28-34 hpf	FKBP5, IκBα, PEPCK up-regulated; IL-8, IL-1β, TNFα down-regulated	Schaaf et al., 2009
zebrafish (*Danio rerio*) embryos	DEX (water soluble)	1000	-	1-3 dpf, then caudal fin amputated	delay in wound healing, massive amount of cell death by necrosis and apoptosis, reduction in number and misplacement of macrophages at the wound site, aberrant migration and misplacement of neutrohpils and macrophages at the wound site	Sharif et al., 2015
zebrafish (*Danio rerio*) embryos	PRE	0.0003, 0.003, 0.03*	EtOH	0-24 hpf 0-48 hpf 0-96 hpf	frequency of spontaneous muscle contraction reduced increased swimming distance and hatching rate after stimulus morphological changes to the lower jaw	McNeil et al., 2016
zebrafish (*Danio rerio*) adult	PRE	25	-	1 d	enhanced osteoclast activity and matrix resorption	de Vrieze et al., 2014
zebrafish (*Danio rerio*) embryos	PRE	10, 25	was used, not specified	5-10 dpf	bone mass reduction	Barrett et al., 2006

Abbreviations

GCs: DEX: dexamethasone, PRE: prednisolone, BDP: beclomethasone dipropionate, CB: clobetasone butyrate, CLOB: clobetasol, CORT: cortisol, CP: clobetasol propionate, BEC: beclomethasone, BV: betamethasone-17-valerate, TRI: triamcinolone, HAL: halcinonide.

Solvent vehicles: DMF: dimethylformamide, DMSO: dimethylsulfoxide, EtOH: ethanol.

Exposure duration: dpa: days post amputation, dpf: days post fertilization, dph: days post hatch, hpf: hours post fertilization.

* studies detected effects close to concentrations predicted or measured in the environment.

1.3 Inflammatory response in zebrafish embryos

The innate immune system of zebrafish is functional by one dpf (Herbomel et al., 1999), while the adaptive immune system is established only several weeks post fertilization (Lam et al., 2004; Trede et al., 2004; Willett et al., 1999). Thus, using the zebrafish embryo model up until 5 dpf for exposure experiments gives the opportunity to examine immune-related effects on the innate immune system alone.

The innate immune system identifies pathogens by recognizing pathogen associated molecular patterns (PAMPs) via pattern recognition receptors (PRRs) (Janeway, 1989). Bacterial lipopolysaccharide (LPS), a constituent of cell walls in Gram-negative bacteria, is a common PAMP recognized by the PRR Toll-like receptor 4 (TLR4) in mammals (Poltorak et al., 1998). When LPS binds to TLR4, a pro-inflammatory cascade is activated, resulting in the release of mediators, like cytokines and chemokines. Myeloid differentiation primary response 88 (MyD88) is the adaptor protein of TLRs. In mammals, LPS-induced inflammation is regulated by MyD88-dependent- and independent pathways (**Fig. 1.3**).

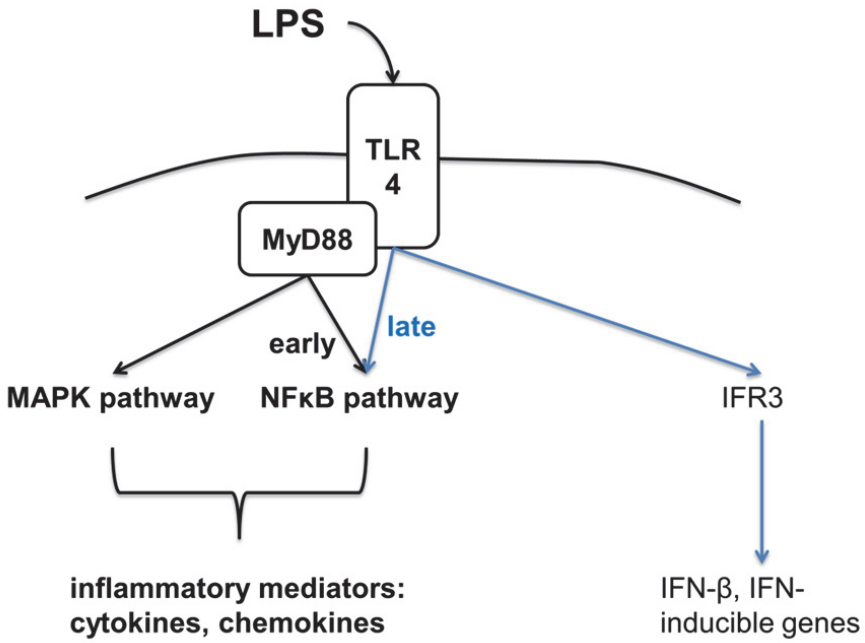

Figure 1.3: Bacterial lipopolysaccharide (LPS)-induced inflammatory response. *When LPS binds to the Toll-like receptor 4 (TLR4), MyD88-dependent and -independent pathways are activated. The dependent pathway (black arrows) induces the early phase activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and the mitogen-activated protein kinase (MAPK) pathways resulting in the release of inflammatory mediators. The independent pathway (blue arrows) involves the late phase activation of NFκB, and also activates the interferon (IFN) regulatory factor 3 (IFR-3), leading to the production of IFN-β and thus expression of IFN-inducible genes (Takeda and Akira, 2004).*

It has already been shown that LPS induces the expression of key inflammatory genes in zebrafish embryos (Mottaz et al., under review; Novoa et al., 2009; Watzke et al., 2007). Indeed, the MyD88-dependent pathway is involved in the innate immune response of zebrafish embryos (van der Sar et al., 2006), thus this pathway is conserved from mammals to fish. Therefore, challenging the embryos with LPS after exposure to GCs and comparing the responses with the untreated control fish allows examining if GCs can suppress the inflammatory response in fish. The molecular mechanism behind the immunosuppressive effect can be investigated by analyzing gene expression patterns of GC-responsive genes and/or inflammatory response-related genes, such as key players in the MyD88-dependent pathway. If genes in these pathways would turn out to be regulated in a robust way after exposure to GCs, they can be used as biomarkers to predict impacts of wastewaters or contaminated freshwaters on fish.

1.4 Finding and validating biomarkers to detect steroidal anti-inflammatory drug effects

Given the ubiquitous presence of GCs in the aquatic environment and their potential impact on fish, bioassays are needed that 1) are fish-based and 2) enable detection of pollutants in environmental water samples that interfere with the stress axis. As mentioned before, biological GR activity is frequently measured by the *in vitro* GR-CALUX®. This bioassay is a human cell line-based reporter gene assay focusing on compounds able to interfere with the GR. Thus, this test is somewhat artificial and does not necessarily identify compounds able to interact with the GR of an aquatic organism, such as fish. Furthermore, it only focuses on GR agonistic or antagonistic compounds, and does not recognize compounds interfering with the HPI axis at other levels.

Examining gene expression on the mRNA and/or protein level, or measuring metabolites after exposure to a potent reference GC in zebrafish embryos, can result in the identification of fish-specific biomarkers. Even though gene expression on the mRNA level is informative, it reflects only the initial step potentially leading to functional proteins. Hence, measurements on the protein and metabolite level give a more integrated understanding of the molecular mechanisms occurring after exposures to single compounds and/or environmental water samples. Selected Reaction Monitoring (SRM) is a targeted proteomics technique utilizing liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) (Lange et al., 2008; Picotti and Aebersold, 2012). This technique has been used in various applications, such as studying the sex differentiation process in zebrafish gonads (Groh et al., 2013), or developing a sentinel protein assay simultaneously monitoring 188 cellular processes in *Saccharomyces cerevisae* exposed to different environmental perturbations (Soste et al.,

2014). Studying the protein expression in zebrafish embryos by SRM is a new and promising area (Groh and Suter, 2014). Alternatively, analyzing the metabolome allows determining the end products of metabolism after a toxic challenge. For instance, a targeted metabolomics method focusing on neurotransmitters has been developed and applied in zebrafish embryos (Tufi et al., 2016a). The same research group also developed a non-targeted metabolomics platform for measuring polar metabolites in the aquatic snail *Lymnaea stagnalis* and used it to measure toxicity of pesticides (Tufi et al., 2015a; Tufi et al., 2015b; Tufi et al., 2016b). Thus, both targeted and global proteomics and metabolomics can be used to explore the downstream response of the zebrafish embryo proteome and metabolome to GC exposure.

Candidate biomarkers have to be specific for a certain mode-of-action (MoA) to minimize interference from chemicals possessing a similar MoA. This is difficult to achieve because of cross-talk between molecular pathways. When a compound group-specific biomarker cannot be identified, biomarkers for a more general or integrative effect, such as indicators of immunosuppression, can be explored. The molecules used as biomarkers need to react significantly after exposure to environmentally relevant concentrations in order to avoid the necessity to enrich environmental water samples, which possibly introduces artifacts. Moreover, the biomarkers need to give a robust response even in a complex matrix, such as environmental water samples.

1.5 Scope of the thesis

Given the omnipresence of GR-active compounds in the aquatic environment and their potential harmful effects on fish, the aim of my research was to assess the effects of GCs on zebrafish embryos, with a particular focus on environmentally relevant concentrations, mechanisms of action and the identification of biomarkers of GC exposure and effects. My PhD project was part of the EDA-EMERGE Marie Curie ITN (EU Initial Training Network), which focused on training young scientists to meet the major challenges in the monitoring, assessment and management of toxicants in the European surface and drinking waters (Brack et al., 2013). During the project, a new generation of EDA approaches was developed for identifying toxicants in European surface and drinking waters by integrating innovative mode-of-action based biodiagnostic tools, including *in vitro* tests, transgenic organisms and 'omics' techniques with powerful fractionation and cutting edge analytical and computational structure elucidation tools (EDA-EMERGE, homepage). The 4th year of my research was funded by the Swiss Federal Office for the Environment (FOEN) in a project focusing on endocrine disrupting compounds, and with the goal to find GC-specific and -sensitive biomarkers of exposure in zebrafish embryos.

A joint European Demonstration Programme (EDP) was conducted within the frame of EDA-EMERGE in four catchments – Sava in Croatia, Danube in the Czech Republic and Slovakia, Saale in Germany, and Emme in Switzerland – providing practical experience in organizing and running international sampling and monitoring campaigns. During the campaigns, the GR-CALUX® *in vitro* bioassay helped identify GR-active sites in all four catchments, including a Swiss site downstream of a municipal WWTP (Tousova et al., in preparation). Based on GCs identified at the Swiss site using a higher tiered EDA (HT-EDA) approach, I subsequently explored the mechanisms of GCs' action in zebrafish embryo as a model.

The overall goals of my thesis research were: 1) to become familiar with the EDA approach and use it to find GR-active compounds in environmental water samples, 2) to investigate the effects of GCs on various physiological processes such as development and the immune system in zebrafish embryos, and 3) to find and validate specific and sensitive biomarkers which can be used in EDA to detect GC-like activity in environmental water samples.

My main steps to achieve these goals were to:

- Explore the GR-active site in Switzerland with HT-EDA using the GR-CALUX® assay combined with an in-house developed targeted GC method (Ammann et al., 2014) in order to find the compounds causing the biological activity. (**Chapter 2;** Sonavane, Schollee and Hidasi et al., in preparation);
- Assess the uptake and inflammatory response in zebrafish embryos on exposure to a highly potent, environmentally relevant GC: clobetasol proprionate (CP). The LPS challenge assay (Mottaz et al., under review) was applied to examine whether CP suppresses the inflammatory response of zebrafish embryos. Furthermore, expression of genes related to GC action and/or the inflammatory response were examined in order to characterize the molecular mechanisms behind the anti-inflammatory effects in fish. (**Chapter 3**; Hidasi et al., under review)
- Identify and validate candidate biomarkers of GC effects and exposure. First, potential biomarkers of anti-inflammatory effects were identified (CP exposure, mRNA level, Chapter 3). The response of these putative biomarkers was also explored after exposure to a non-steroidal anti-inflammatory drug, diclofenac (DCF, mRNA level) for comparison. A targeted proteomics method was developed for some of these genes and additional genes identified from the literature (40 GC-action related proteins). Of these, those target genes detected in control embryo digests were further analyzed (12 proteins detected in zebrafish embryos) after CP and DCF exposures. Finally, the candidate biomarkers (mRNA, protein) were validated with environmental grab water

samples containing GCs and DCF (HT-EDA site). (**Chapter 4**; Hidasi et al., in preparation)

- Investigate the metabolome of zebrafish embryos after CP exposure using global metabolomics (Tufi et al., 2015a) and targeted metabolomics focusing on neurotransmitters (Tufi et al., 2016a). This part of the work was conducted at IVM, Vrije Universiteit, Amsterdam during my secondment in the frame of EDA-EMERGE (**Chapter 5**).

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Chapter 2:

Identification of endocrine disrupting chemicals in urban waste-water effluent using effect-directed analysis combining passive sampling, mechanism based *in vitro* and *in vivo* bioassays and chemical analyses

Wastewater treatment plant (WWTP) effluents are considered major sources of endocrine disrupting chemicals (EDCs) in the aquatic environment. In view of the hazard associated with these compounds, their identification and monitoring in WWTPs is required in order to limit risk for aquatic ecosystems. In this study, we assessed the impact of a WWTP effluent in a river using a battery of bioassays to monitor endocrine activities and embryo toxicity and identify active chemicals in an effect-directed analysis (EDA) approach. *In vitro* screening revealed the WWTP effluent discharge site as a source of estrogens, glucocorticoids, arylhydrocarbon receptor (AhR) agonists and other toxic contaminants. Most of the activities observed were the highest at the effluent discharge point and to a lesser extent at a downstream site. In addition, endocrine activities and developmental effects were also monitored using zebrafish embryo assays, i.e. the zebrafish embryo toxicity test (FET) and transgenic (cyp19a1b-GFP) zebrafish embryos. The fractionation of the crude extract and *in vitro* testing of the fractions showed a clear separation of estrogenic and glucocorticoid receptor active chemicals. This allowed the identification of potent estrogens (estrone and 17β-estradiol) and glucocorticoids (clobetasol propionate and fluticasone propionate) using LC-MS/MS analysis.

Keywords: WWTP effluent, EDCs, Chemcatcher® passive sampler, EDA

2.1 Introduction

Endocrine disrupting chemicals (EDCs) are exogenous substances that alter the functioning of hormonal systems and produce a range of developmental, reproductive, neurological, and immunological effects in humans and wildlife (Hotchkiss et al., 2008). The sources of EDC exposure are diverse and vary widely around the world, but effluents of wastewater treatment plants (WWTPs) are considered one of the major sources of discharge into the aquatic environment (Ihara et al., 2015; Jarosova et al., 2014). These effluent discharges represent a threat for aquatic organisms. For instance, exposure to WWTP effluents containing (xeno)-estrogens has been associated with the impairment of reproduction and sexual differentiation of aquatic organisms (Lange et al., 2012; Routledge and Sumpter, 1996; Sanchez et al., 2011). In addition to estrogenic contaminants, occurrence of other natural and synthetic steroids have been reported and linked to discharge from urban, industrial or hospital WWTPs (Chang et al., 2007; Schriks et al., 2010; Van der Linden et al., 2008). These steroids include androgens, corticosteroids or progestogens that can interfere with the regulation of the endocrine system by binding to steroid receptors such as the androgen (AR), glucocorticoid (GR) and progesterone receptor (PR). The presence of these compounds in the environment at environmental concentrations in the ng/L range (measured or predicted) represents a risk to aquatic organisms (Besse and Garric, 2008; Runnalls et al., 2010). Altogether these findings highlight a growing need to monitor multiple EDCs and their combined effects at WWTP effluent discharge sites for a better ecotoxicological assessment of water bodies.

Characterization of environmental contamination by EDCs requires the use of proper sampling devices that provide a representative picture of water contaminants. This is especially true in view of their wide polarity range, often leading to partitioning into different compartments, and the dynamics of the contaminant load (Vermeirssen et al., 2006). Grab or composite samples provide single time points or an averaged time period only, with restrictions in terms of low detection limit. Passive sampling has become a promising alternative for obtaining a time-integrated evaluation of the bioavailable contaminants in river waters and to realistically assess exposure of aquatic organisms (Vrana et al., 2005 ; Vermeirssen et al., 2005). In combination with *in vitro* assays, this approach allows the detection of mixtures of bioactive chemicals present at very low concentration levels.

Mixture effects have been considered one of the key challenges in ecotoxicological research (Eggen et al., 2004) and quite some effort has gone into developing and implementing reliable and sensitive monitoring strategies (Ruff et al., 2015). Regulation-based environmental monitoring uses target chemical analysis and provides a quantitative assessment of already known chemicals with high selectivity and sensitivity. However, it

does not provide comprehensive information on chemical contamination and thus very often fails to explain observed biological effects (Brack et al., 2016). To address such issues, mechanism/effect-based tools have been proposed. For instance, a wide variety of *in vitro* assays, i.e. mechanism-based tools, currently exists for the assessment of EDCs. They are considered a relevant and promising option to specifically address hazards to human and ecosystems (Grimaldi et al., 2015). Many studies have reported their use for water quality assessment, in particular for monitoring the chemical quality of WWTP effluents (Creusot et al., 2014a; Macikova et al., 2014; Jia et al., 2016). More recently, mechanism based *in vivo* tools have been developed that allow the integral detection of EDCs by taking into account the pharmaco-kinetics of contaminants at the whole organism level (Brion et al., 2012). In particular, *in vivo* fish tools have been adopted as an environmentally relevant strategy for the testing of chemicals (OECD, 2013). Furthermore, fish embryo tests have successfully been used to assess water chemical quality using effect-directed analysis (EDA) (Fetter et al., 2014; Legler et al., 2011).

If monitoring of endocrine disrupting activities of WWTP effluents is relevant in regard to the environmental risk of EDCs, the chemical identification of the compounds responsible for these activities is even more crucial in order to propose measures for the reduction of their emission into aquatic ecosystems, such as substitution of EDCs by non-active compounds or advanced wastewater treatment (e.g. ozonation, sorption to active carbon). EDA that combines biotesting, fractionation and chemical analysis addresses this issue by separating active chemicals into different fractions, reducing the sample complexity in the process and with that ion suppression (Brack, 2003). In fact, since the last decade, several studies have highlighted the usefulness of this approach for the identification of EDCs in environmental matrices, in particular by using passive samplers (Creusot et al., 2014a; Liscio et al., 2009; Liscio et al., 2014).

In the present study, we implemented the effect-directed analytical strategy by combining passive sampling (Chemcatcher®), mechanism-based bioassays and chemical analyses for the monitoring of WWTP effluent and up- and downstream locations. We selected sites near a WWTP along the River Urtenen in Switzerland, where we recently reported the occurrence of estrogens, glucocorticoids and other contaminants (Tousova et al., in preparation). In this work, we first performed *in vitro* screening using reporter gene assays based on different nuclear receptors in order to establish the endocrine disrupting profile of the investigated WWTP. *In vivo* assays were then applied to confirm endocrine disruption at the whole organism level. Finally, the more active extracts (effluent) were fractionated in order to isolate active chemicals and to identify them by using cutting-edge liquid chromatography coupled to mass spectrometry (LC-MS).

2.2 Materials and methods

2.2.1 Chemicals and reagents

The chemical standards 17β-estradiol, dihydroxy-testoterone (DHT), flutamide, dexamethasone (DEX), 2,3,7,8-TCDD, benzo[a]pyrene (BaP) and 3,4- dichloroaniline (DCA), the solvent dimethyl sulfoxide (DMSO) and enzyme substrates (7-ethoxy-resorufin and luciferin, 7-ERF) used for bioassays were all purchased from Sigma Aldrich (France). Dichloromethane (DCM) and methanol (MeOH) of HPLC reagent grade were purchased from VWR (France). The chemical standards for the targeted GC analysis (betamethasone, budesonide, clobetasol, clobetasol propionate, corticosterone, cortisone, dexamethasone, dexamethasone-21-acetate, flumetasone, fluorometholone, fluticasone propionate, hydrocortisone, medroxyprogesterone, 6α-methylprednisolone, mifepristone, prednisolone, prednisone, triamcinolone acetonide, aldosterone, eplerenone, fludrocortisone-21-acetate, 21-hydroxyprogesterone, progesterone, spironolactone, bicalutamide, cimetidine, clotrimazole, daidzein, fluconazole, genistein, glycyrrhetinic acid, ketoconazole, metyrapone, miconazole, β-naphtoflavone, pravastatin, quercetin and resveratrol) were purchased from Sigma-Aldrich (Switzerland). Standard solutions were prepared in ethanol and stored at -20 ºC. HPLC-gradient pure acetonitrile (ACN) was from Acros Organics (Chemie Brunschwig AG, Switzerland).

2.2.2 Study sites, sampling and extraction procedures

The river Urtenen is a tributary of the river Emme in Switzerland. The WWTP is located along the river Urtenen into which it discharges treated effluent. Downstream of the point of discharge the river contains up to 40% treated wastewater (**Figure 2.1**).

In the present study, we investigated the occurrence of EDCs from the WWTP by using the passive sampler Chemcatcher® (SDB discs covered by PES membranes). For this purpose, three sites were sampled between September and October 2014, two located upstream and downstream of the WWTP respectively and one directly downstream of the effluent pipe of the WWTP (**Figure 2.1**). Ten Chemcatcher® discs were deployed at each of the three locations for a period of 4 weeks, after which they were stored in vials with acetone at 4^{o}C until further extraction and analysis. The Chemcatcher® discs were then extracted as described previously (Moschet et al., 2015). Briefly, each disc was extracted in 6 mL of acetone followed by 6 mL of methanol. The acetone fraction was dried to approximately 1 mL and later combined with the methanol fraction. All extracts were pooled and aliquoted for biological and chemical analyses. The amount of water enriched in the sampler was expressed with an enrichment factor in L.EQ/L (based on average sampling rate of 0.288 L/day and deployment of 14 days). For biological analyses, extracts were taken up in DMSO.

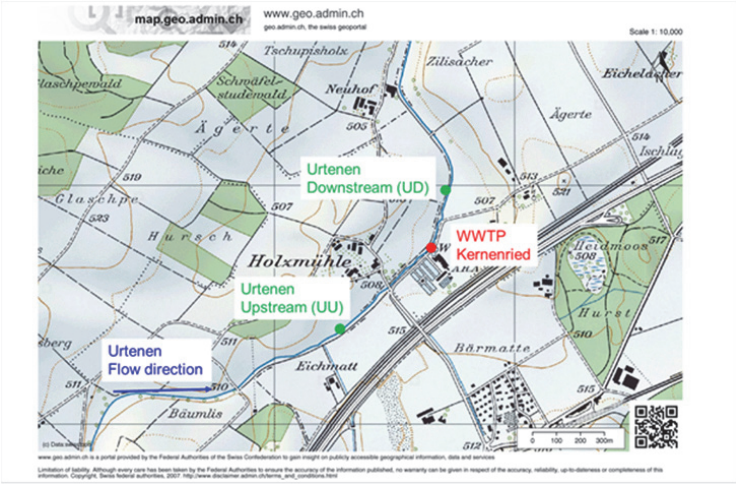

Figure 2.1: *Map of the Urtenen river site. The three sampling sites were located upstream (UU), near the WWTP effluent discharge point (red dot) and downstream (UD).*

2.2.3 *In vitro* assays for EDCs detection

We used a battery of well-established mechanism-based *in vitro* assays that allows the detection of estrogenics (MELN, Balaguer et al., 1999; zfERα and zfERβ2, Cosnefroy et al., 2012), androgenics (MDA-kb2, Wilson et al., 2002), glucocorticoids (MDA and GR-CALUX®, Van der Linden et al., 2008) and dioxin-like chemicals (PLHC-1, Louiz et al., 2008). All these assays are based on cell line cultures that stably express a reporter gene (luciferase) under transcriptional control of a nuclear receptor. Conditions of routine culture and exposure of these cell lines have been described in detail previously (Cosnefroy et al., 2012; Creusot et al., 2014a; Van der Linden et al., 2008; Macikova et al., 2014). The cells were exposed to serial dilutions of extracts, solvent controls (DMSO) and reference compounds (positive control), and were finally processed for luciferase activity. The EROD (7-ethoxyresorufin-O-deethylase) assay was used to assess AhR activation, using PLHC-1 cells that were exposed for 4 h (PAH-like activity) and 24 h (dioxin-like activity). For the MDA-kb2 assay, co-exposure experiments were performed in order to confirm glucocorticoid or androgenic activity using the GR-antagonist RU486 and the AR-antagonist flutamide. For the GR-CALUX® assay, dexamethasone was used as a positive control.

2.2.4 Zebrafish based *in vivo* assays

The zebrafish (*Danio rerio*) embryo acute toxicity test (FET) (OECD, 2013) and the EASZY tg(cyp19a1b-GFP) (Brion et al., 2012) assays were used to assess acute toxicity and estrogenic activities, respectively. Zebrafish maintenance and breeding has been carried out as previously explained (Creusot et al., 2014b). Fertilized eggs were selected at 0 days post fertilization (dpf) for both *in vivo* assays and exposed to environmental extracts.

For the **FET** assay, 20 embryos were placed in a 24-well plate, each with one embryo per well in 1 mL of test media (294.0 mg/L $CaCl_2{\cdot}2H_2O$, 123.3 mg/L $MgSO_4{\cdot}7H_20$, 63.0 mg/L $NaHCO_3$ and 5.5 mg/L KCl) and 4 embryos as internal plate control. In addition, 20 embryos were also used as solvent control (DMSO; 0.1 v/v %) and positive control (DCA; 4 mg/L). Exposures were performed from 0 to 4 dpf in an incubator at 28 ºC with a light/dark cycle of 14 h/10 h, under semi-static conditions (50% renewal). Apical observations for lethal and sub-lethal effects were recorded every 24 h after the start of exposure, until the end of the test at 96 h. The observations used to determine lethality included: coagulation of embryos, lack of somite formation, non-detachment of the tail and lack of heartbeat (< 1 beat/ min).

In addition, the **EROD** assay was performed on 4 dpf exposed zebrafish at the end of the exposure period, when each zebrafish larva was manually placed onto white 96-well microplates containing 200 µL of water supplemented with 7-ERF 5 µM for fluorescence measurement (Fluotrac 200, Grenier, France). Kinetics of resorufin production was directly measured in living zebrafish for 20 hours with a spectrofluorophotometer thermostated at 27 ºC (Saphire II, Tecan, Switzerland).

For the **EASZY** assay, organic environmental extracts were tested as previously described (Sonavane et al., 2016). Briefly, each experimental group consisted of 20 embryos exposed in 15 mL of re-circulated water in a separate glass petri dish. The used solvent concentration (DMSO, v/v 0.1%) did not affect the GFP fluorescence in the tg(cyp19a1b-GFP) embryos. Exposures period and conditions used were the same as for the FET assay with daily renewal of 50 % of environmental extract and removal of dead embryos, if any. After the end of the exposure period (96 h), live (tg-cyp19a1b-GFP) larvae were individually observed in dorsal view and the brain was photographed to measure GFP expression using a Zeiss AxioImager.Z1 fluorescence microscope equipped with a AxioCam Mrm camera (Zeiss, GmbH, Germany). Details of the parameters used and image analysis performed have been explained previously (Brion et al., 2012).

2.2.5 Biological and chemical toxic equivalents calculation

In vitro and *in vivo* dose-response curves were calculated using the Hill model in the Excel Macro Regtox (freely available at http://www.normalesup.org/~vindimian/). This allows calculation of the 20 % effect concentrations (EC_{20}) of the tested samples by fixing the slope, and the minimum/maximum effect values to that of the reference compound (e.g. E2 for MELN assay) (Villeneuve et al., 2002). Bioassay-derived toxic-equivalents (bio-TEQs) for hormone-like or dioxin-like activities were determined as the ratio of the EC_{20} of the reference chemical expressed as g/L to that of the environmental sample expressed as enrichment factor in L.EQ/L (Creusot et al., 2014a).

2.2.6 HPLC fractionation

In order to isolate the compounds responsible for the observed estrogenic and glucocorticoid activities, the effluent extract was fractionated using HPLC. This was achieved with a reverse phase (RP) fractionation on a C18 column (Poursuit C18, 5 µm, 250 X 4.6 I.D, Varian, France) that has been previously calibrated for the isolation of chemicals covering a broad range of polarities (Creusot et al., 2013). Briefly, the run was performed at 25 ^{o}C with a flow rate of 1 mL/min using a water/acetonitrile gradient program: 0-10 min (80/20), 60 min (55/45), 100-120 min (0/100), 120-125 min (80/20). The collected fractions (3 mL) were dried using an EZ-2 evaporator system and then redissolved in 200 µL of ACN for storage until biological and chemical analyses.

2.2.7 Targeted chemical analysis

Targeted chemical analysis for estrogens and glucocorticoids was carried out for the active fractions using LC-MS/MS as described previously (Liscio et al., 2009; Ammann et al., 2014). The active fractions were received in aliquots of approximately 28 µL ACN. Upon arrival, they were diluted to ca. 120 µL with MeOH. From this, 30 µL was used for the targeted estrogenic and 30 µL for the targeted GC analysis. For the GC analysis, these aliquots were evaporated down to ca. 13 µL under a gentle stream of N_2, and 27 µL milliQ water was added to each. 10 µL were injected from this mixture and analyzed as described in Ammann et al., 2014.

2.2.8 Quality control

Blanks were prepared at each step for quality control. At the study sites, a sampling blank was prepared using the Chemcatcher® SDB samplers, prior to sampling. For biotesting, solvent blanks were prepared and run in parallel with our samples. A fractionation blank was also prepared to identify any possible contamination during fractionation, showing up in the bioassays. All the tested blanks were found to be negative in the bioassays.

2.3 Results and Discussion

2.3.1 Toxicological profiling

2.3.1.1 In vitro profiling of endocrine disrupting activities

In vitro screening of the Urtenen river sites for endocrine disrupting activities revealed the WWTP effluent discharge site as a likely source of estrogenic, glucocorticoid and dioxin-like activities. Most of these activities observed were the highest at the effluent discharge point and to a lesser extent at downstream and upstream sites (**Table 2.1**).

For estrogenic activity, E2-EQ values were measured by the hERα (human) and zfERβ2 (zebrafish) in vitro assays. The activities determined in the hERα and zfERβ2 assays were 0.13 and 0.10 ng EQ/L for upstream, 0.16 and 0.19 ng EQ/L for downstream, and 0.48 and 0.54 ng EQ/L for effluent sites, respectively. Interestingly, no estrogenic activity was detected at all the three sampling points by the zfERα assay. In regard to androgenic and gluco-corticoid activities assessed through the MDA-kb2 assay, our results showed no androgenic but strong glucocorticoid activity when co-exposed with RU486 and flutamide. At the WWTP effluent and downstream sites, the MDA-kb2 measured DEX-EQ values were found to be 534 and 86 ng EQ/L, respectively. The presence of glucocorticoid activity was further confirmed using the GR-CALUX® assay. Higher levels of DEX-EQs (**Table 2.1**) were detected in effluent extract (46.3 ng EQ/L) which were comparable to data reported in other field studies (Creusot et al., 2014a; Macikova et al., 2014; Van der Linden et al., 2008). It is worth to be noted that the glucocorticoid activity quantified as DEX-EQs was 10-12 fold higher in the MDA-kb2 than the GR-CALUX® assay. Such differences may be due to specificity and sensitivity of the assays towards different GR ligands, metabolic capacity or transcriptional interference as is the case with AR expression in the MDA-kb2 (Wilson et al., 2002). Finally, using the PLHC-1 cell line, dioxin-like activity was also detected at all the three sites, indicating a contamination by AhR ligands with the effluent being slightly more active than the other sites. Since this activity is detected after both 4 h and 24 h exposure duration, the samples are contaminated by both readily metabolized chemicals such as polycyclic aromatic hydrocarbons and also more persistent contaminants such as polychlorinated biphenyls, polychlorinated dibenzo-dioxins/-furans. The measured TCCD-EQ and BaP-EQ values ranged between 0.4-0.7 ng EQ/L and 83-261 ng EQ/L, respectively. The occurrence of estrogenic, glucocorticoid and dioxin-like activities in the effluent and to a lesser extent in the downstream site confirmed the effluent as a source of contaminants.

Table 2.1: Summary of the *in vitro* toxicological profile of the Urtenen River sites

Activity	Receptor (cell line assay)	Urtenen (ng EQ/L)		
		Upstream	Effluent	Downstream
Estrogenic E2-EQ	Human ERα (MELN)	0.13 (0.10-0.15)	0.48 (0.38-0.62)	0.16 (0.13-0.18)
	Zebrafish ERα (ZELH-α)	n.d.	n.d.	n.d.
	Zebrafish ERβ2 (ZELH-β2)	0.10 (0.08-0.12)	0.54 (0.41-0.81)	0.19 (0.15-0.23)
Androgenic DHT-EQ	Human AR/GR (MDA-kb2)	n.d.	n.d.	n.d.
Gluco-corticoid DEX-EQ	Human GR/AR (MDA-kb2)	n.d.	534.1 (515.3-592.7)	85.8 (78.2-88.0)
	Human GR (GR-CALUX)	n.d.	46.3 (32.5-50.5)	9.5 (8.8-9.7)
Dioxin-like TCDD-EQ	(PLHC-1)	0.40 (0.36-0.45)	0.67 (0.59-0.78)	0.52 (0.45-0.59)
PAH-like BaP-EQ	(PLHC-1)	83.4 (67.2-109.6)	261.3 (200.4-373.1)	104.8 (82.0-164.9)

Results are expressed as estradiol (E2-EQ) / dihydrotestosterone (DHT-EQ) / dexamethasone (DEX-EQ) / 2,3,7,8-tetrachloro-dibenzodioxin (TCDD-EQ) / benzo[a]pyrene (BaP-EQ) equivalents in ng EQ/L with the 95% confidence interval in brackets derived from the dose response curves presented in Fig. S2.1; n.d.: not detected (<LOQ).

2.3.1.2 In vivo effects for hazard confirmation

Using zebrafish embryos, we characterized lethal and endocrine disrupting activities at an early developmental stage of aquatic species (**Table 2.2**). Using the FET assay, toxicity was observed at the WWTP effluent discharge site to a 7-fold higher extent, i.e. LC_{50} of 9 L EQ/L (sample enrichment by the Chemcatcher® and dilution in the assay), as compared to the downstream site, whereas, very low toxicity was observed at the upstream site (**Table 2.2, Table S2.1, Figure S2.2**).

Table 2.2: Summary of *in vivo* zebrafish embryo assays at the Urtenen River sites

Activity	*In vivo* assays	Urtenen (ng EQ/L or L.EQ/L)		
		Upstream	Effluent	Downstream
Lethality LC_{50}	FET	>100	9	63
Estrogenic **E2-EQ**	Tg(*cyp19a1b*-GFP) (EASZY)	0.7	19.6	8.5
Dioxin-like **TCDD-EQ**	FET (EROD)	0.10	0.68	0.13

LC_{50}: lethal concentration affecting 50% of the total population, expressed as enrichment factor in L.EQ/L. E2-/TCDD-EQ: estradiol-/2,3,7,8-tetrachloro dibenzo dioxin-equivalent expressed as ng.EQ/L. EROD activity on zebrafish larvae measured after 96 hours of sample exposure.

In parallel, sub-lethal and teratogenic effects were also monitored and quantified at distinct stages as reported previously (Fraysse et al., 2006; Nagel, 2002). Among the three sites, the effluent site showed a high percentage of concentration-dependent increases for specific abnormalities at 48 hours post fertilization (hpf) prior to the observed toxic effects after 72 and 96 hpf. For the downstream site with non-toxic concentrations, similar abnormalities were observed at 96 hpf. Hence, prolonged exposure to the downstream site extract might potentially result in increased mortality of the exposed embryos due to chemicals released from the effluent site. Knowing that this WWTP effluent discharge is from municipal sources (households and industries), a diverse group of micropollutants is potentially responsible for such lethal and sub-lethal effects, as previously reported for personal care products (Brausch and Rand, 2011) and pharmaceuticals (Johnson et al., 2015; Zhang et al., 2015). In addition, a recent study documented that PAH-like compounds are a cause of developmental toxicity in zebrafish (Wincent et al., 2015). In our study, we quantified higher PAH-like activities by the *in vitro* assay and thus could link the observed lethal and sub-lethal effects on zebrafish embryos to these compounds.

Using the tg(cyp19a1b-GFP) assay, *in vivo* estrogenic potential was detected at all three sites in the developing brain of 96 hpf old transgenic zebrafish (**Figure 2.2**). The GFP expression induced by the effluent site was found to be significant at 10 L.EQ/L. At the downstream site, similar GFP induction was measured at 3-fold higher concentration, i.e. 30 L.EQ/L, while the upstream site showed lowest estrogenic activity in this assay. Using the mean EC_{20} values, the quantified E2-EQ values at all three sites ranged between 0.7 ng EQ/L and 19.6 ng EQ/L (**Table 2.2**). As expected from the *in vitro* data, the order of *in vivo* potential based on the quantified E2-EQ values demonstrated similar estrogenic profile, i.e.

effluent > downstream > upstream (**Table 2.2**). For estrogenic activity, the mechanism-based *in vivo* (EAZSY) assay confirmed the effects of the *in vitro* detected ER activity, adding ecotoxicological relevance. The comparison of *in vitro* and *in vivo* E2-EQs revealed significant differences at all three sites that showed higher estrogenic activity by the *in vivo* assay. For example, the *in vivo* assay showed 36, 45 and 7 fold increases in E2-EQ values compared to the zebrafish *in vitro* assay (zfERβ2) for effluent, downstream and upstream sites, respectively. This could be due to the presence of corticoids, or other sex steroids and their transformation products that have previously been reported to produce ER-mediated responses (Liu and Shi, 2015). Also, Brion et al. (2012) showed high *in vivo* estrogenic activity by synthetic progesterones (norethindrone and levonorgestrel) but no activity by progesterone itself. Moreover, the metabolic capacity of the *in vivo* assay could also contribute to the observed higher *in vivo* E2-EQ values. This is further supported by the 50 % renewal of the extract every 24 h for the *in vivo* exposure, resulting in higher exposure concentration of the ER ligands as compared to the *in vitro* assay.

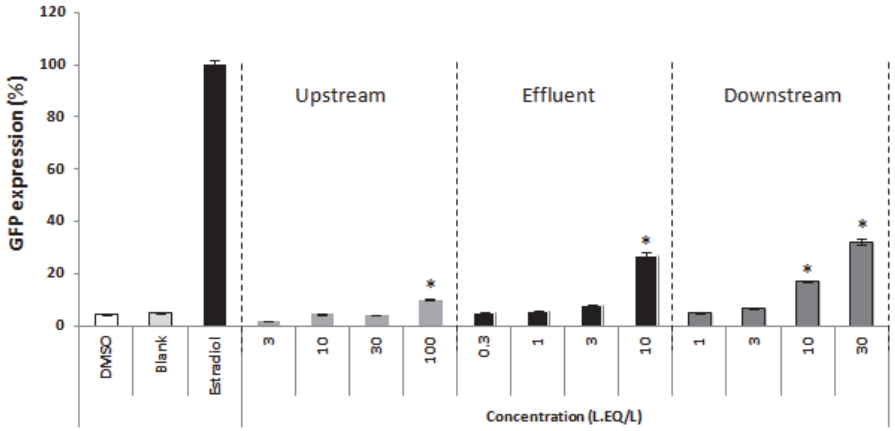

Figure 2.2: *In vivo* estrogenic profile of the Urtenen River sites on zebrafish larvae using transgenic cyp19a1b-GFP line. *(n=20 number of larvae exposed per condition). X axis: non-toxic concentrations expressed as enrichment factor in L.EQ/L used with semi-static renewal (50 % sample renewal every 24 hours). *: $p < 0.05$*

In addition, a mechanism-specific endpoint was assessed to detect AhR ligands. Measurement of EROD activity on living zebrafish larvae revealed the presence of AhR ligand activity at all the three sites (**Table 2.2, Figure S2.3**). Further quantification of TCDD-EQ values confirmed similar AhR activity profiles as depicted by the PLHC-1 cells. Knowing the ability of AhR ligands or their metabolites to activate estrogen receptors (Kummer et al., 2008; van Lipzig et al., 2005; Ohtake et al., 2003) or interfere with ER-signaling pathways as antagonist (Cheshenko et al., 2007) could also contribute to the observed estrogenic effect *in vivo* and thus explain the above differences to measure estrogenic activity.

2.3.1.3 Risks for aquatic ecosystem

Risk assessment requires to link exposure to hazard. In this study, we combined sample enrichment with the SDB-Chemcatcher®, with a panel of *in vitro* and *in vivo* assays for the detection of a broad range of chemical contaminants. Here, we show the presence of multiple EDCs and toxicants in the effluent and adjacent sampling sites, confirming the suitability of this combined approach for monitoring different pollutants in environmental water bodies, which is in line with what has been recently reviewed (Lissalde et al., 2016). Knowing the capacity of the Chemcatcher® to retain more polar compounds, i.e. log K_{ow} 2-6 (Schafer et al., 2008; Vermeirssen et al., 2009), we had the certainty to enrich polar estrogenics, and glucocorticoids that would not have been caught with a classical C18 SPE phase alone (**Table 2.1**). However, concentrations reported by the Chemcatcher® sampler do not quantitatively reflect actual water concentration due to different temperature and flow dependent sampling rates (R_s) of individual chemicals, ranging from 0.0002 to 0.4 L/day across 88 determined compounds (Moschet et al., 2015). Until now, R_s have not been described for the above detected agonists using Chemcatcher® passive sampler (Moschet et al., 2015; Vermeirssen et al., 2013). By using a median R_s of 0.07 L/day (Moschet et al., 2015), the effect concentration in the *in vitro* assays, calculated based on sampling rate dependent concentration and bio-TEQs, are 0.3 ng E2-EQ/L, 273 ng DEX-EQ/L (MDA-kb2), 24 ng DEX-EQ/L (GR-CALUX®) ,0.4 ng TCDD-EQ/L and 133 ng BaP-EQ/L in the effluent and 0.1 ng E2-EQ/L, 44 ng DEX-EQ/L (MDA-kb2), 5 ng DEX-EQ/L (GR-CALUX®), 0.3 ng TCDD-EQ/L and 53 ng BaP-EQ/L at the downstream site. By using *in vivo* assays, these values were 10 ng E2-EQ/L and 0.4 ng TCDD-EQ/L at the effluent site, and 4.3 ng E2-EQ/L and 0.06 ng TCDD-EQ/L at the downstream site.

It is well known that steroidal estrogens can lead to estrogenic effects at measured environmental concentrations (Caldwell et al., 2012). It is noteworthy that the above E2-equivalents determined *in vitro* in water at our studied sites were lower than the environmental quality standard (EQS) value for surface water proposed by the EU Water Framework Directive [i.e. 0.4 ng/L (SCHER, 2011)]. However, estradiol equivalents were found to be higher than 0.4 ng/L at both effluent and downstream sites in the *in vivo* assay (**Table 2.2**), which reflects a true physiological response in an intact organism. Thus the estrogenic activity detected at the studied river sites has the potential to affect fish populations by inducing feminization in some species of male fish (Purdom et al., 1994), causing transgenerational effects on survival and fecundity (Schwindt et al., 2014), abnormal reproduction (Brion et al., 2004) and abnormal secondary sexual characteristics (Lange et al., 2012).

Unlike estrogens, there is very limited information on the effects of glucocorticoids on freshwater fish, even though higher quantities may reach the aquatic environment in comparison to the usually investigated (xeno)estrogens (Creusot et al., 2014a; Runnalls et al., 2010). In our study, the total glucocorticoid concentration was in the ng/L range in rivers. This can potentially alter reproductive or developmental functions in fish (Leatherland et al., 2010), or cause immunosuppression (Chapter 3; Margiotta-Casaluci et al., 2016).

In summary, our results suggest that the detected and identified EDCs at the downstream river site could affect well-being and reproduction of fish, which stresses the need to increase our understanding on the effects of estrogens and glucocorticoids on aquatic organisms. Furthermore, it highlights the need to better characterize and identify these compounds in complex mixtures that interfere with crucial signaling pathways in order to predict and limit their impacts on fish health.

2.3.2 Identification of active compounds in the effluent by EDA approach

Toxicological profiles of the Urtenen river sites by a panel of bioassays revealed, not very surprisingly, the effluent as being the most biologically active site, containing multiple contaminants. To isolate the active contaminants, the effluent extract has been fractionated using HPLC to yield 40 fractions of decreasing polarity that were tested using different *in vitro* assays. The RP-HPLC profile of the effluent extract showed well separated estrogenic and glucocorticoid activities pattern (**Figure 2.3**).

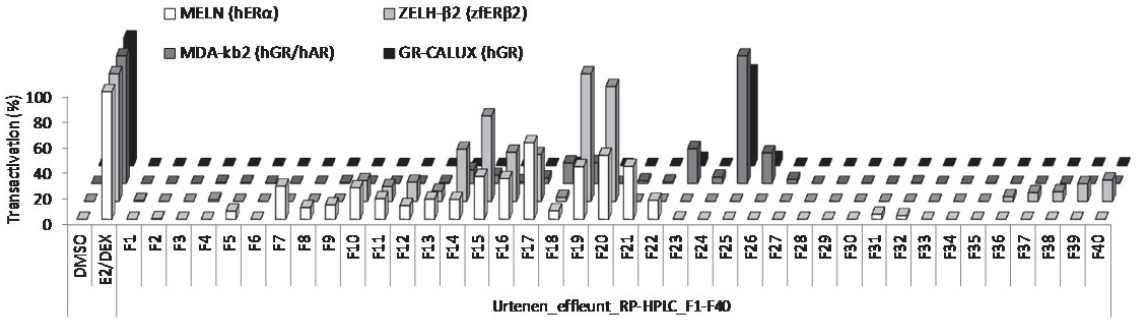

Figure 2.3: RP-HPLC profiles of Urtenen effluent extract showing estrogenic (MELN and ZELHβ2) and glucocorticoid (MDA-kb2 and GR-CALUX®) activity.

Using zebrafish and human *in vitro* assays, we observed similar estrogenic patterns from fractions F10 to F21. Interestingly, some estrogenic fractions, i.e., F14, F15, F19 and F20 showed higher activity in the zfERβ2 than hERα-based *in vitro* assay, including the most active one (F19). Some weakly active fractions, i.e., F7 and F21 showed specificity towards hERα but not to zfERβ2. Such differences in the response could be explained by the

specificity of the assay towards different estrogenic ligands due to differences across species (Cosnefroy et al., 2012; Rastall et al., 2006). It was reported that the fraction of compounds captured by the passive sampler are the freely dissolved that are taken up by many aquatic species, highlighting the importance of fish-specific assays for risk assessment of aquatic organisms. For GR ligands, both MDA-kb2 and GR-CALUX® assays showed strong activity in F25, with other less active fractions (F23 and F26). In addition, we observed some weakly active fractions with the MDA-kb2 assay (i.e. F14, F18 and F19) that were not detected by the GR-CALUX® assay. This could be attributed to the presence of androgenic compounds that might have interfered with the glucocorticoids in the whole extract. Overall, these results highlight the relevance of fractionation to reduce the complexity and isolate active chemicals for further identification.

To identify the active chemicals in the active fractions, target chemical analyses of estrogens and glucocorticoids was performed using LC-MS/MS (Ammann et al., 2014). Target analysis of the estrogenic fractions revealed the presence of known estrogens, with the highest concentration of E2 and E1 in some of the most active fractions (F16, F17 and F19).

Further High Resolution Mass Spectrometry (HRMS)-based non-targeted analyses of the estrogenic fractions will allow identification of unknown fish-specific ER ligands. This will further help to better characterize toxicants for hazard and risk for aquatic species at studied river waters.

Target chemical analysis of the GR-active fractions F23, F25 and F26, revealed different classes of compounds (**Table 2.3**). In the two less active fractions, F23 and F26 the bio-TEQ values based on the GR-CALUX® results were 16.2 and 14.9 ng/mL DEX-EQs, respectively. No known GR-active compound was quantifiable in any of these fractions. Therefore, these two fractions should be subjected to non-target chemical analyses in order to find the compounds responsible for the biological activity. In the most active fraction (i.e. F25), pravastatine, clobetasol propionate and fluticasone propionate were found in higher concentrations. Pravastatine was found to be not GR-active (Macikova et al., 2014). Previous field studies have described glucocorticoids as being continuously released by WWTP effluents (Ammann et al., 2014; Chang et al., 2007; Macikova et al., 2014; Schriks et al., 2010; Van der Linden et al., 2008). Based on the high bio-TEQs of clobetasol propionate and fluticasone propionate (Macikova et al., 2014) measured by the GR-CALUX® assay, the chem-TEQ value was calculated to be 275.8 ng/mL DEX-EQs. The bio-TEQ value of this fraction was 321.8 ng/mL DEX-EQs. Thus, the detected clobetasol propionate and fluticasone propionate concentrations explained 85.7% biological activity (**Table 2.3**).

Table 2.3: Detected compounds using targeted GC analysis (Ammann et al., 2014) and Mass Balance Analysis of chemical and biological activities of the GR-active fractions

F#	Name	Class	REP GR-CALUX®	Concen-tration (ng/mL)	Chem-TEQ (ng/mL)	Bio-TEQ (ng/mL)	Mass Balance chem/bio
F23	fluorometho-lone	GC	0.98	<LOQ	-	16.2	0%
F25	clobetasol propionate	GC	38	5.4	205.8	321.8	87.0%
	fluticasone propionate	GC	57	1.3	74.2		
	progesterone	progesto-gen	NA	<LOQ	-		
	mifepristone	GR antagonist	anta-gonist	<LOQ	-		
	ketoconazole	anti-fungal	ND	<LOQ	-		
	pravastatin	cholesterol synthesis inhibitor	ND	8.1	-		
F26	β-naphto-flavone	AhR agonist	NA	1.6	-	14.9	0%
	pravastatine	cholesterol synthesis inhibitor	ND	<LOQ	-		
	glycyrrhetinic acid	11β-HSD1/2 inhibitor	ND	37.5	-		

NA: not assessed for GR activity, ND: no GR activity detected

Our findings identified these compounds as the main contributors to the GR agonistic activity in the whole effluent extracts. These observations are in line with previous reports where clobetasol propionate and fluticasone propionate significantly contributed to the prediction of glucocorticoid activity in sewage treatment plant effluents (Macikova et al., 2014; Suzuki et al., 2015). It was also reported that the presence of such synthetic glucocorticoids may contribute strongly to the total activity of effluents even at lower effluent concentrations. However, it should be noted that the occurrence of these potent glucocorticoids contributing to GR agonistic activity are location-specific. For instance, triamcinolone acetonide was reported as major contributor to the GR activity in effluent extracts from the Netherlands (Schriks et al., 2010).

2.4 Conclusions

In the present study, we determined the contamination of Urtenen river waters by a mixture of EDCs through the combined use of SDB Chemcatcher® and mechanism-based bioassays. At this river site, we showed the WWTP discharge to be a major source of multiple endocrine disrupting compounds and anthropogenic contaminants including ER, GR and AhR agonists. Along with the lethal and sub-lethal effects on zebrafish embryos, *in vivo* confirmation of estrogenic and dioxin-like activities observed in *in vitro* assays highlighted the hazard for aquatic organisms and thus raises the question of an associated risk for aquatic ecosystems. Further investigations involving fractionation, *in vitro* assays and chemical analyses (i.e. EDA) allowed identifying potent estrogens (E1, E2 and EE2) and glucocorticoids (clobetasol propionate and fluticasone propionate). Additionally, given the fact that different species display different sensitivity towards chemicals, the use of human derived cell lines could represent a limitation when extrapolating risk of environmentally relevant concentrations of EDCs to aquatic organisms. Such differences could be overcome by using fish-derived cell lines as reported in this study. In this context, we also demonstrated that mixtures of environmental contaminants do not necessarily exhibit the same activity *in vivo* as *in vitro* (i.e. estrogenic activity), suggesting the need to combine *in vitro/in vivo* approaches for improving hazard and risk assessment of EDCs in aquatic ecosystems. In the present study, such a comparison was not performed in regard to glucocorticoids, but this could possibly be addressed by the use of the recently developed GRIZLY assay (Weger et al., 2012). Furthermore, physiological effects of GCs on aquatic organisms can also be investigated by identifying potential GC-sensitive protein biomarkers in fish using targeted proteomics (Chapter 4). In the future, implementation of such new and advanced species-specific bioassays combined with instrumental analysis will allow more accurate assessment of the risk associated with EDCs in aquatic complex mixtures.

Contributions: I took part in the passive sampler deployment, collection and extraction together with Jennifer Schollée. The GR-CALUX® assays were performed by Nadine Bramaz, and I evaluated the data. The targeted GC analysis was run by Adrian Amman, and I carried out the data evaluation with the help of René Schönenberger. I was writing up the GC-related part of the manuscript.

2.5 Supplementary information

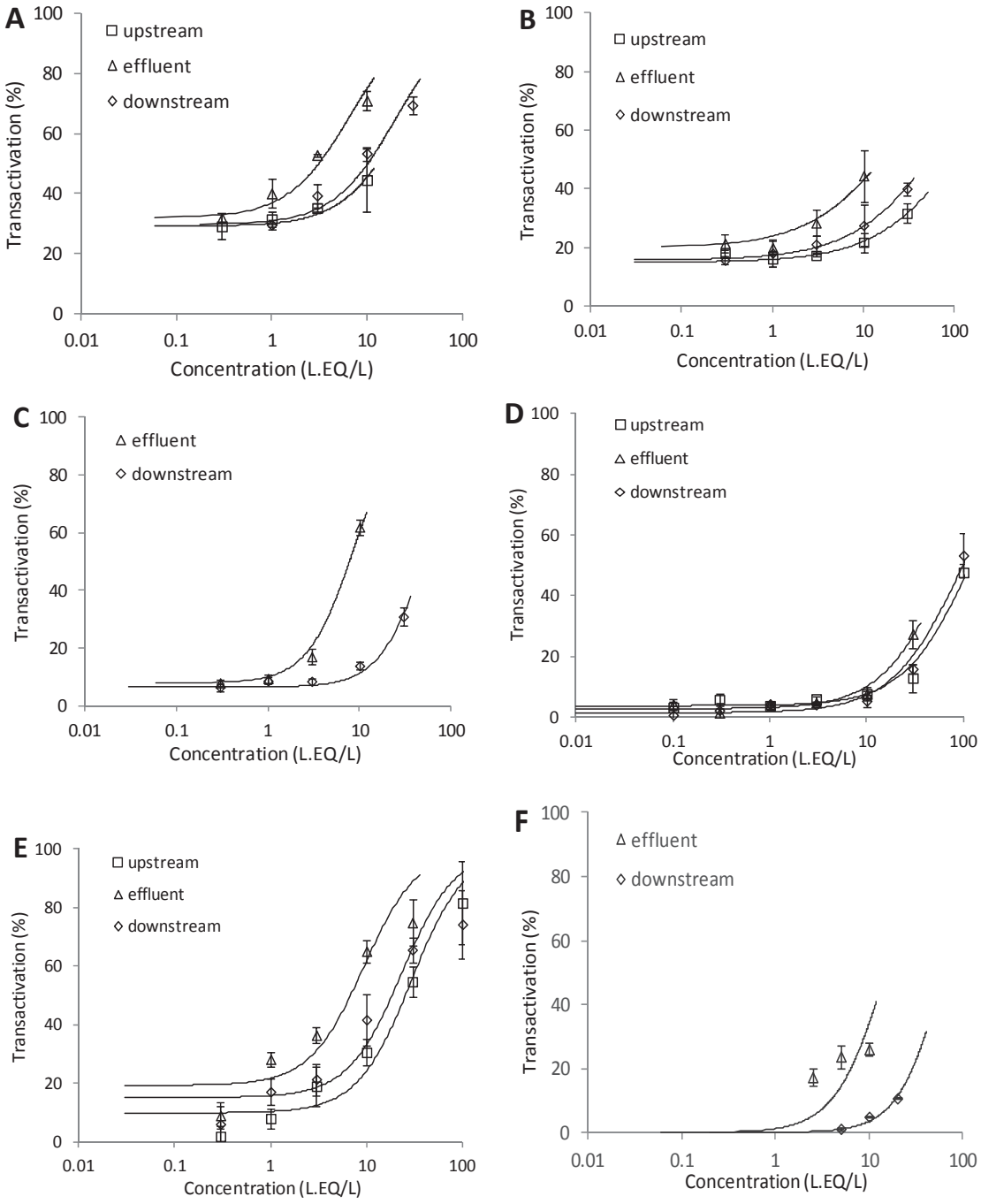

Figure S2.1: Dose-response curves of luciferase induction in (A) MELN-hERα, (B) ZELH-zfERβ2, (C) MDA-kb2, (D and E) PLHC-1 for dioxin-like and PAH-like activities and (F) GR-CALUX® cell lines exposed to Urtenen river sites.
Concentrations are expressed as enrichment factors in L.EQ/L.

Table S2.1: Summary of acute toxicity of Urtenen river sites using FET assay.

	Cumulative effects at 96 hpf		
Sample dilutions in L.EQ/L	**Upstream**	**Effluent**	**Downstream**
100	30%	100%	80%
30	30%	90%	10%
10	30%	40%	0%
3	10%	30%	0%
1	10%	20%	0%
0.3	30%	0%	0%
LC50	> 100	9	63
LC20		6	39

Lethality is expressed as percentage based on 4 endpoints: coagulation, no heartbeat, no somite formation and no head-tail detachment; $LC_{50/20}$: lethal concentration affecting 50 % or 20 % of the total population, expressed as enrichment factor in L.EQ/L.

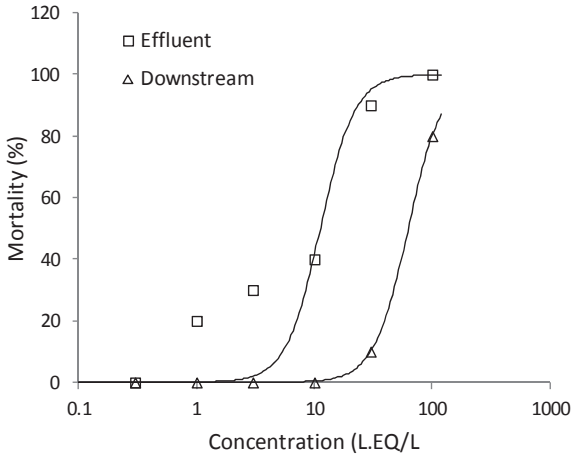

Figure S2.2: Dose-response curves of Urtenen river sites showing acute toxicity. *Results are expressed as percentage of mortality of zebrafish embryos. LC_{50} values are derived by fixing the slope to 2.98 and maximum of 100%.*

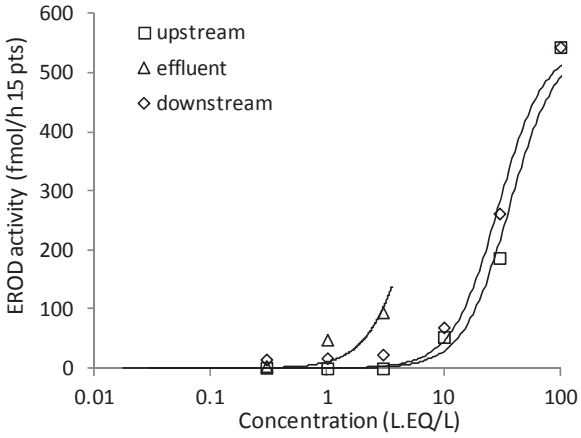

Figure S2.3: Dose response curves of Urtenen river sites showing EROD activity on zebrafish larvae. *Results are expressed as fmol/h of zebrafish larvae. EC_{20} values are derived by fixing the slope to 2.22 and maximum to 544 fmol/h.*

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Chapter 3:

Clobetasol propionate causes immunosuppression in zebrafish (*Danio rerio*) at environmentally relevant concentrations

Synthetic glucocorticoids (GCs) are potential endocrine disrupting compounds that have been detected in the aquatic environment around the world in the low ng/L (nanomolar) range. GCs are used as immunosuppressants in medicine. We were interested whether clobetasol propionate (CP), a highly potent GC, suppresses the inflammatory response in fish after exposure to environmentally relevant concentrations. Bacterial lipopolysaccharide (LPS) challenge was used to induce inflammation and thus mimic pathogen infection. Zebrafish embryos were exposed to ≤1000 nM CP from 0 day post fertilization (dpf) to 4 dpf, and CP uptake, survival after LPS challenge, and expression of inflammation-related genes were examined. Our initial experiments were carried out using 0.001% DMSO as a solvent vehicle, but we observed that DMSO interfered with the LPS challenge assay, and thus masked the effects of CP. Therefore, DMSO was not used in the subsequent experiments. The internal CP concentration was quantifiable after exposure to ≥10 nM CP for 4 days. The bioconcentration factor (BCF) of CP was determined to be between 16 and 33 in zebrafish embryos. CP-exposed embryos showed a significantly higher survival rate in the LPS challenge assay after exposure to ≥0.1 nM in a dose dependent manner. This effect is an indication of immunosuppression. Furthermore, the regulation pattern of several genes related to LPS challenge in mammals supported our results, providing evidence that LPS-mediated inflammatory pathways are conserved from mammals to teleost fish. *Anxa1b*, a GC-action related anti-inflammatory gene, was significantly down-regulated after exposure to ≥0.05 nM CP. Our results show for the first time that synthetic GCs can suppress the innate immune system of fish at environmentally relevant concentrations. This may reduce the chances of fish to survive in the environment, as their defense against pathogens is weakened.

Keywords: teleost fish, endocrine disrupting chemicals, pharmaceuticals, inflammation, bacterial lipopolysaccharide

3.1 Introduction

Synthetic glucocorticoids (GCs) are commonly used in human and veterinary medicine for their immunosuppressive effects. GCs mimic the action of the steroid hormone cortisol, the natural stress hormone in humans and fish. They activate the glucocorticoid receptor (GR) and thus regulate the expression of GR-regulated genes playing a role in the immune system, but also development, glucose metabolism, osmoregulation, bone formation and behavior. Thus, GCs are potential endocrine disruptors: due to their hormone-like mode-of-action they may disturb the homeostasis of the stress-axis, i. e. the hypothalamus-pituitary-interrenal (HPI) axis in fish, and the hypothalamus-pituitary-adrenal (HPA) axis in mammals.

Kugathas and coworkers reviewed GC concentrations detected in the aquatic environment up to 2012, and used a mathematical model to predict the total environmental concentration of GCs in the River Thames (Kugathas et al., 2012). Based on their review and calculations, they proposed to conduct laboratory experiments assessing GC effects on aquatic organisms in the range between 10 and 1000 ng/L total GC. Indeed since then, several studies confirmed the presence of GCs around the world. The total detected GC concentrations were up to 57 ng/L in surface waters, up to 100 ng/L in wastewater treatment plant (WWTP) effluents and up to 836 ng/L in WWTP influents (Ammann et al., 2014; Herrero et al., 2014; Iglesias et al., 2014; Isobe et al., 2015; Jia et al., 2016; Liu et al., 2015; Macikova et al., 2014; Nakayama et al., 2016; Suzuki et al., 2015). Most of these studies used analytical procedures that focus on individual compound detection.

GR-activation potential of single chemicals, chemical mixtures and environmental water samples can also be assessed using an *in vitro* assay named GR-CALUX®, which measures the total ability of the compounds present in a sample to bind to and activate the GR (Van der Linden et al., 2008). The potency of individual GCs to activate the GR varies, and it is expressed in relative potencies (REP) normalized to dexamethasone. Application of this and similar assays detected GR-activity up to 2.7 ng/L dexamethasone equivalents (DEX-EQs) in surface waters and up to 155 ng/L DEX-EQs in WWTP effluents (Creusot et al., 2014; Jia et al., 2016; Macikova et al., 2014; Schriks et al., 2013; Stavreva et al., 2012; Suzuki et al., 2015; Van der Linden et al., 2008). Moreover, based on this approach, clobetasol propionate (CP) was shown to be one of the most potent GCs, having a REP of 38 (Macikova et al., 2014). CP itself was detected in many studies cited above at concentrations up to 1 ng/L in surface waters, 4.9 ng/L in WWTP effluents, and 7 ng/L in untreated wastewater (Ammann et al., 2014; Isobe et al., 2015; Jia et al., 2016; Macikova et al., 2014; Nakayama et al., 2016; Suzuki et al., 2015). Hence GCs, such as CP, can reach the aquatic environment in significant concentrations and potentially adversely affect the aquatic biota. Fish and

amphibians are most likely to be affected due to the conservation of major GR targets across vertebrates (Schaaf et al., 2009; Schoonheim et al., 2010; Steenbergen et al., 2011).

The results obtained in most of the studies examining GC effects in fish may have limited relevance for environmental risk assessment because much higher exposure concentrations than found in the aquatic environment were used (Gadan et al., 2012; Mathieu et al., 2013; Philip et al., 2012; Philip and Vijayan, 2015; Salas-Leiton et al., 2012; Sharif et al., 2015). Moreover, most reported studies used dimethyl sulfoxide (DMSO) as solvent carrier even though DMSO is known to have side-effects that may interfere with the endpoint of the assay used, and thus potentially affect the results (Oliveira et al., 2016; Santos et al., 2003). In this study, we attempted to address these aspects, using zebrafish embryo, an excellent model for GC research (Alsop and Vijayan, 2009; Schaaf et al., 2009).

The innate immune system of zebrafish is active by one day post fertilization (dpf) (Herbomel et al., 1999), whereas the adaptive immune system is fully developed only 4-6 weeks post fertilization (Lam et al., 2004; Trede et al., 2004; Willett et al., 1999). Thus, using the zebrafish embryo model until 120 hours post fertilization (hpf) for exposure experiments gives the opportunity to examine immune-related effects on the innate immune system alone. Moreover, using the zebrafish embryo model until 120 hpf, i. e. during the non-protected life stage, is considered an alternative to conventional animal testing (Strahle et al., 2012).

One of the important ways by which the innate immune system identifies pathogens is by recognizing pathogen associated molecular patterns (PAMPs) via pattern recognition receptors (PRRs) (Janeway, 1989). Bacterial lipopolysaccharide (LPS), a constituent of cell walls in Gram-negative bacteria is a common PAMP recognized by the PRR Toll-like receptor 4 (TLR4) in mammals (Poltorak et al., 1998). When LPS binds to TLR4, a pro-inflammatory cascade is activated, resulting in the release of mediators, like cytokines and chemokines. Myeloid differentiation primary response 88 (MyD88) is the adaptor protein of TLRs. In mammals, LPS-induced inflammation is regulated by MyD88-dependent and -independent pathways. The dependent cascade regulates the early phase activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and the mitogen-activated protein kinase (MAPK) pathways resulting in the release of inflammatory mediators, while the independent pathway involves the late phase activation of NFκB, furthermore it also activates the interferon (IFN) regulatory factor 3 (IFR-3) that is leading to the production of IFN-β and thus expression of IFN-inducible genes (Takeda and Akira, 2004). It has already been shown that LPS induces the expression of key inflammatory genes in zebrafish embryos (Mottaz et al., under review; Novoa et al., 2009; Watzke et al., 2007). Indeed, the MyD88-dependent pathway is involved in the innate immune response of zebrafish embryos (van der Sar et al., 2006), thus it is a conserved pathway. Hence, challenging the embryos

with LPS after exposure to a model GC and comparing the responses with the untreated control fish allows investigating whether GCs can cause immunosuppression in fish.

The aim of this study was to assess whether CP, an environmentally relevant GC that has a high potency to activate the GR, causes immunosuppressive effects in zebrafish embryos. The uptake of CP into embryos was also measured, and the bioconcentration factor (BCF) of CP for zebrafish embryos was determined. Gene expression analyses of genes involved in the anti-inflammatory action of GCs and/or the MyD88-dependent pathway were performed in order to investigate the molecular mechanism behind the observed inflammatory effects.

3.2 Materials and methods

3.2.1 Chemicals

Clobetasol propionate (CP, CAS 25122-46-7), clobetasol (CAS 25122-41-2), hydrocortisone (CAS 50-23-7), lipopolysaccharide from *Pseudomonas aueruginosa*, NH_4HCO_3, dimethyl sulfoxide (DMSO), and the salts used to prepare the dilution water (294 mg/L $CaCl_2$ x 2 H_2O, 123.2 mg/L $MgSO_4$ x 7 H_2O, 64.74 mg/L $NaHCO_3$, and 5.75 mg/L KCl; prepared according to OECD test guideline 236) were purchased from Sigma-Aldrich (Buchs, Switzerland). The dilution water was prepared in MilliQ water. D9-progesterone (Progesterone-2,2,4,6,6,17a,21,21,21-d9, CAS 15775-74-3) was purchased from CDN Isotopes (Dr. Ehrenstorfer, Augsburg, Germany). Ethyl-acetate was from Merck (Zug, Switzerland), and HPLC-gradient pure acetonitrile (ACN) was from Acros Organics (Chemie Brunschwig AG, Basel, Switzerland).

3.2.2 Zebrafish husbandry and embryo collection

Wild type embryos with mixed genetic background from WiK (Max Planck Institute for Developmental Biology, Tübingen, Germany), OBI (Helmholtz Centre for Environmental Research established from OBI hardware store, Leipzig, Germany) and Qualipet (petshop, Switzerland) were used in this study. Zebrafish were maintained according to recommended procedures (Nüsslein-Volhard and Dahm, 2002). Adult zebrafish were kept in a Mass Embryo Production System (Aquatic Habitats®, Pentair Aquatic Eco-Systems, Apopka, FL, USA) with a mix of tap and reversed-osmosis water (1:1) using a 14/10 h light/dark cycle at 28 °C. The embryo collection system was placed in the tank in the afternoon the day before collection. The embryos were collected 45 min after the light turned on in the morning, washed, and kept in dilution water (prepared according to OECD guideline 236). Next, the fertilized embryos were selected and placed in the pre-soaked wells or petri dishes, depending on the experiment type.

3.2.3 Exposures

Approximately 0.5-1 mg CP was dissolved in one liter of dilution water and the concentration was measured by a UV-Vis spectrophotometer (Cary 100, Agilent Technologies, Basel, Switzerland) at 240 nm wavelength. The stock solution was kept at 4 °C up to one week. Embryos were exposed to ≤1000 nM CP (≤ 46.7 µg/L) from 0 dpf to 4 dpf at 28 °C using a 14/10 h light/dark cycle. For the uptake measurements, embryos were kept in small petri dishes (d=60 mm), with 15 mL exposure solution and 30 embryos per dish. In the initial LPS challenge assays, CP was first dissolved in DMSO and then spiked into the dilution water. Final DMSO concentration in these experiments was 10 µL/L, i. e. 0.001 % (v/v). For the LPS challenge assay, embryos were kept in 6-well microtiter plates with 5 mL exposure solution and 12 embryos per well. For qPCR analyses, 24 embryos in 12 mL were raised in small petri dishes. Before the experiments, the respective exposure vessels were pre-soaked with the exposure solutions for one day. Only 70 % of the exposure solutions were changed every day during the experiments in order to avoid stressing the embryos.

3.2.4 CP concentration in exposure medium and in fish

Uptake measurements were carried out with embryos exposed to 0, 1, 10, 100 and 1000 nM CP. CP was extracted according to the procedure described by Yeh and coworkers for cortisol (Yeh et al., 2013), since cortisol is structurally similar to CP. 25-30 embryos were anaesthetized by ice-cold dilution water, collected and snap-frozen in liquid N_2 at 96 hpf. After homogenization of the samples with an ultrasonic processor (LABSONIC® M, Sartorius Stedim Biotech GmbH, Goettingen, Germany; parameters: cycle 1, amplitude 100%, 3x10 sec, with 20-30 sec breaks, all on ice), 1 ng D9-progesterone was spiked into the samples as internal standard (IS) and the homogenate extracted with ethyl-acetate. After evaporation, samples were dissolved in 50 µL acetonitrile (ACN). In order to monitor the CP concentration, 500 µL samples were taken during one of the exposure experiments before and after changing the exposure solution. Cortisol, CP and clobetasol were measured in the embryo extracts and exposure medium samples by an LC-MS/MS selected reaction monitoring (SRM) method developed earlier (Ammann et al., 2014). Briefly, 10 µL sample were injected using a PAL autosampler. LC separation was achieved on a Poroshell EC C18 column (100x2.1 mm, 1.7 µm, Agilent) using a 20 min water/ACN gradient with 5 mM NH_4HCO_3 at 200 µL/min flowrate. Two SRM transitions per analyte were monitored in positive mode electrospray (Vantage, ThermoFisher Scientific), m/z 363 → 121 / 327 for cortisol, m/z 467 → 355 / 373 for CP, m/z 411 → 373 / 391 for clobetasol, and m/z 324 → 100 / 113 for D9-progesterone. The first transitions were used for quantitation and the second as qualifiers. Needle voltage was set to +3000 V, vaporizer temperature to 150 °C, capillary temperature to 250 °C. Collision energy was 25 V for cortisol, 20 V for CP and clobetasol,

and 30 V for D9-progesterone. The concentrations were calculated from the area under the quantifier peaks using calibration standard curves. The detected CP concentrations were normalized to the internal standard in the samples, and then normalized to the mean wet weight of the embryos. Limit of detection and limit of quantitation (LOD and LOQ) values of CP were calculated according to the 1:3 and 1:10 signal-to-noise ratio concept. In the exposure media, the signal-to-noise ratio of the control samples were averaged and the LOD and LOQ values were calculated based on it. In this case, LOD was 50 ng/L, and LOQ was 225 ng/L. In case of the embryo samples, the signal-to-noise values of the control embryo extracts were averaged and used for the calculation of the limits of the method. The LOD was 2.5 ng/g wet tissue, and the LOQ was 5 ng/g wet tissue.

3.2.5 LPS challenge assay

Embryos were exposed to 0.001, 0.01, 0.1, 1, 10 and 100 nM CP. The LPS challenge assays were performed according to Mottaz et al (Mottaz et al., under review). At 81 hpf, embryos were plated with 100 µL exposure solution onto 96-well plates (10-12 embryos/concentration/plate). Embryos were treated with *P. aeruginosa* LPS (19-21 µg/mL) at 97 hpf. Survival of the embryos was monitored each hour starting at 2 hours post challenge (hpc) until 10 hpc, then the next day at 23 hpc using a Leica CTR 6000 inverted microscope. Results were plotted using a Kaplan-Meier survival plot and evaluated with the log-rank test (GraphPad Prism 5).

3.2.6 Gene expression analysis with quantitative RT-PCR (qPCR)

Zebrafish embryos were exposed to 0, 0.001, 0.01, 0.05, 0.01, 0.1, 1, 10 and 100 nM CP. At 96 hpf they were anaesthetized and snap-frozen as described in section 3.2.4. RNA was isolated using the Qiagen RNeasy Mini Kit according to the manufacturer's protocol (Qiagen AG, Hombrechtikon, Switzerland). The isolated RNA concentration was measured by a Nanodrop® ND-1000 spectrophotometer (Nanodrop, USA). RNA was treated with DNase I (DNase I Amplification Grade, Invitrogen by Life Technologies) in order to remove possible genomic DNA contamination. 1 µg purified RNA was reverse transcribed to cDNA (PrimeScript™ RT Reagent Kit, Takara Bio Inc., Japan) in 20 µL volume using a temperature program starting with incubation at 37 °C for 15 min, then at 85 °C for 5 min, finally cooling down to 4 °C (Bio-Rad Laboratories DNA Engine Dyad™). In parallel, 1 µg RNA in RNase free water was ran without the reaction mix for negative controls. For the qPCR analyses, the reaction mix (FastStart Universal SYBR Green Master (Rox), Roche Diagnostics GmbH, Mannheim, Germany) with 25 ng cDNA in triplicates, 25 ng RNA negative controls in duplicates, and water controls in duplicate were pipetted onto 96-well plates (ABI MicroAmp® Fast Optical 96-well Reaction Plate with Barcode, 0.1 mL) using a QIAgility pipetting robot

(Qiagen AG, Hombrechtikon, Switzerland). The reaction volume was 20 µL. The qPCR reactions (ABI 7500 Fast Real-Time PCR System) started with a holding stage at 50 °C for 2 min, then another holding stage at 95 °C for 10 min. 40 cycles were ran (each 95 °C for 15 sec, followed by 57 °C for 1 min). Melting curves were also analyzed (95 °C for 15 sec, 60 °C for 1 min, then measuring the fluorescence of the amplicons by slowly increasing the temperature with 1 % slope up to 95 °C, finally cooling down to 60 °C for 15 sec). The primers used are listed in the supplementary information (**SI Table 3.1**). The mean normalized expression (MNE) values were calculated by normalizing to the expression levels of a housekeeping gene, eukaryotic translation elongation factor 1 alpha 1 (ef1α), then each MNE was divided by the control MNE. The obtained fold inductions compared to control were plotted on the graphs. One-way ANOVA followed by Dunnett's test was used to evaluate whether the differences were statistically significant (GraphPad Prism 5).

3.3 Results

3.3.1 Uptake

The organism-internal concentrations were determined after 0-4 dpf exposure, and they were correlated with the exposure concentrations (**Fig. 3.1, Table 1**). Approximately 70 % of the exposure medium was exchanged daily, and CP concentrations monitored during one of the experiments. The actual CP concentrations in the fresh and 1-day-old medium were determined for each nominal concentration (**SI Fig. 3.1**), and the values averaged over the whole time series were used as mean exposure concentrations for calculating the BCF values (**Table 3.1**). The amount of CP in embryos exposed to 1 nM CP was below the detection limit. CP was detected in embryos exposed to ≥10 nM CP. **Table 3.1** also shows the BCF values calculated by dividing the organism-internal concentrations by the respective mean exposure concentrations. The BCF values ranged between 16 and 33. The LC-MS/MS method also monitored clobetasol, another GC used in medicine, which compared to CP, lacks the propionate group and thus might be its metabolite. However, this potential metabolite was not detected in the exposure medium or in the embryo extracts. Furthermore, cortisol was also monitored, but not detected in any of the samples.

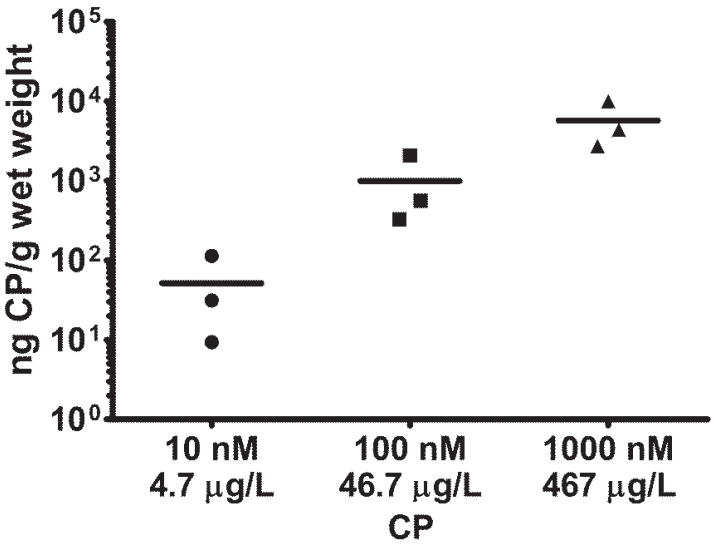


Figure 3.1: Uptake of clobetasol propionate into zebrafish embryos. *The embryos were exposed 0-4 days post fertilization (N=3 independent experiments). Each symbol represents one independent experiment at the respective concentration obtained from 25-30 pooled embryos. The lines represent the mean concentrations detected in the fish.*

Table 3.1: Clobetasol propionate exposure concentrations and bioconcentration factors.

	Nominal exposure concentration (µg/L)	**Mean measured exposure concentration (µg/L)**	**Mean internal concentration in embryos (µg/kg)**	**BCF=C_e/C_w[1]**
control	0	<LOD	<LOD	-
1 nM	0.5	0.3	<LOD	-
10 nM	4.7	3.2	51.4	16.2
100 nM	46.7	30.4	990.2	32.5
1000 nM	466.7	204.7	5694.6	27.8

[1] The BCFs were calculated according to the formula $BCF=C_e/C_w$, where C_e is the CP concentration in the fish embryos, and C_w is the CP concentration in the exposure media.

3.3.2 LPS challenge assay

3.3.2.1 Inflammatory responses to LPS after CP exposure with DMSO as a solvent vehicle

Our initial experiments were carried out using 0.001 % DMSO as a solvent vehicle for CP (**Fig. 3.2**). By the end of the experiment, 34 ± 7 % blank control, but only 7 ± 3 % DMSO control embryos survived. Consequently, the embryos exposed to DMSO control showed significantly higher mortality in the LPS challenge assay than the blank controls. Embryos exposed to CP with DMSO showed the following survival rates by 23 hpc: 5 ± 3 % in the 1 nM CP group, 15 ± 5 % in the 10 nM CP group, 13 ± 5 % in the 100 nM CP group and 15 ± 5 % in the 1000 nM CP group. These groups showed the same statistical differences compared to the blank control, but no significant changes in survival compared to the DMSO control embryos.

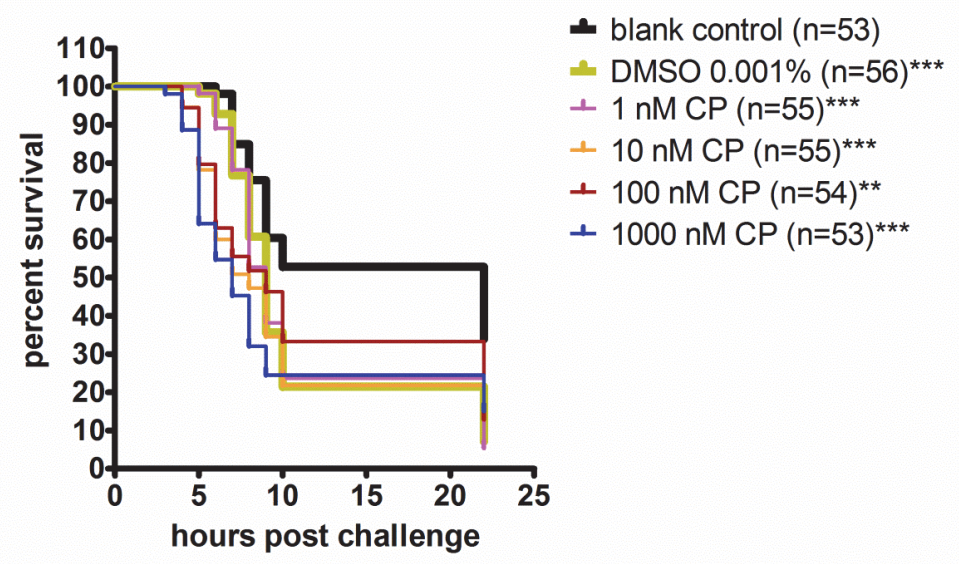


Figure 3.2: Influence of clobetasol propionate with DMSO as a solvent vehicle on the survival of zebrafish embryos after bacterial lipopolysaccharide challenge. *The embryos used in these experiments had genetic backgrounds mixed from WiK and OBI zebrafish. The embryos were exposed to 0.001 % DMSO and 1, 10, 100, 1000 nM CP with 0.001 % DMSO until 4 days post fertilization, then challenged with LPS at 97 hours post fertilization. The survival rate was monitored hourly from 2 to 10 hours post challenge (hpc), and also at 23 hpc. The results are plotted as Kaplan-Meier survival curves (N=3 independent experiments, total number of embryos (n) per concentration point indicated in the graph; ** p<0.005, *** p<0.0005, Log-rank test compared to blank control.) CP-exposed groups were also examined compared to the DMSO group but did not show significant differences.*

3.3.2.2 Inflammatory responses to LPS after CP exposure without DMSO

In these experiments, CP was dissolved in the embryo medium directly. A higher amount of LPS was used, thus the blank control embryos showed a lower survival rate than in the experiments using DMSO. The LPS challenge assay showed significantly higher survival of the CP-exposed embryos starting from 0.1 nM (**Fig. 3.3**). The effect in CP-exposed embryos compared to the blank control group was observed in a concentration-dependent manner. While only 7 ± 3 % of control embryos survived by 23 hpc, 22 ± 5 % embryos survived in the 0.1 nM CP exposure group. The immunosuppressive effect of CP was the strongest after exposure to 10 and 100 nM, where the final survival rates were 68 ± 6 and 65 ± 6 %, respectively. Zebrafish embryos with a different genetic background showed the same trend in response to CP exposure (**SI Fig. 3.3**), with significantly higher survival rates detected at 0.1 nM CP.

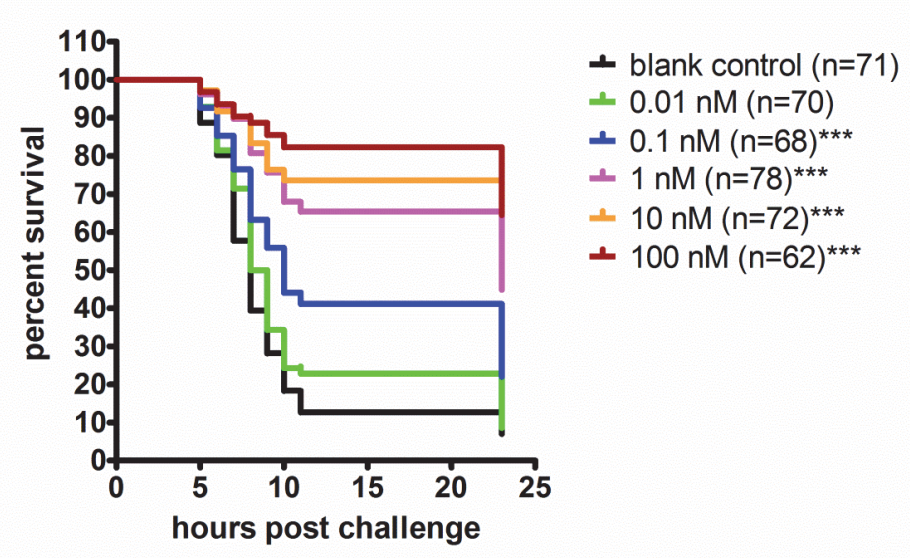
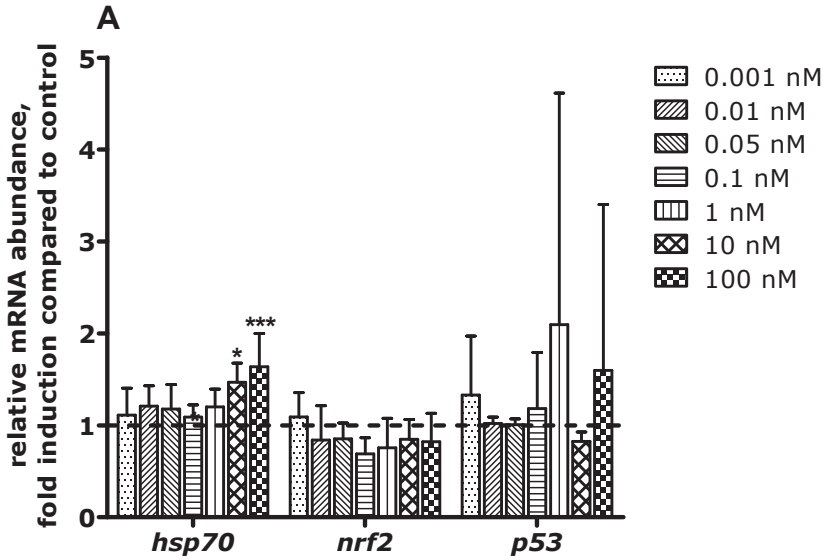


Figure 3.3: Influence of clobetasol propionate without DMSO vehicle on the survival of zebrafish embryos after bacterial lipopolysaccharide challenge. *The embryos used in these experiments had genetic backgrounds mixed from WiK, OBI and Qualipet zebrafish. The embryos were exposed to 0.01, 0.1, 1, 10, 100 nM CP dissolved directly in the dilution water until 4 days post fertilization, then challenged with LPS at 97 hours post fertilization, and the survival rate was monitored from 2 to 11 hours post challenge (hpc) hourly, and at 23 hpc. The results are plotted as Kaplan-Meier survival curves (N=3 independent experiments, total number of embryos (n) per concentration point indicated on the graph; *** p<0.0005, Log-rank test compared to blank control).*

3.3.3 Gene expression of inflammatory response-related genes

The expression of a panel of 10 inflammation-related genes in zebrafish embryos was analyzed after exposure to CP until 4 dpf in order to investigate the molecular mechanisms underlying the strong immunosuppressive results observed in the LPS challenge assay.

The expression of heat shock protein 70 (*hsp70)* was significantly up-regulated after exposure to 10 and 100 nM CP (**Fig. 3.4A**). Nuclear factor erythroid 2-related factor 2 (*nrf2*) and tumor protein p53 (*p53)* showed no significant regulation after CP exposure (**Fig. 3.4A**). Myeloid differentiation primary response 88 (*myd88*), the adaptor protein of TLR4 was significantly down-regulated due to CP exposure at 0.1 and 1 nM CP (**Fig. 3.4B**). The two subunits a and b of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (*IκBα)*, a GR-target gene (De Bosscher et al., 2003) were analyzed. The results for both subunits showed the same trend: no regulation up to 1 nM CP, up-regulation after exposure to 10 and 100 nM CP. The effect was statistically significant only for the b subunit at 10 nM and 100 nM CP (**Fig. 3.4B**). Mitogen-activated protein kinase (MAPK) 14a (one of the four p38 MAPKs, *p38*) was significantly down-regulated after exposure to 100 nM CP (**Fig. 3.4B**). The expression of the v-fos FBJ murine osteosarcoma viral oncogene homolog Ab (*cfos*) showed significant and strong increase at 100 nM CP (**Fig. 3.4B)**. The pro-inflammatory chemokine (C-X-C motif) ligand 8a (interleukin-8, *il8*) that is also a GR-target (Luecke and Yamamoto, 2005), was down-regulated in a dose-dependent way and became significant after exposure to 100 nM CP, while interleukin 10 (*il10*), an anti-inflammatory cytokine showed a trend of up-regulation (**Fig. 3.4C**). Annexin A1b (*anxa1b*)*,* a GR-target (Sengupta et al., 2012) phospholipase A2-inhibitor, was significantly down-regulated in a concentration-dependent manner after exposure to 0.05, 0.1, 1, 10 and 100 nM CP.



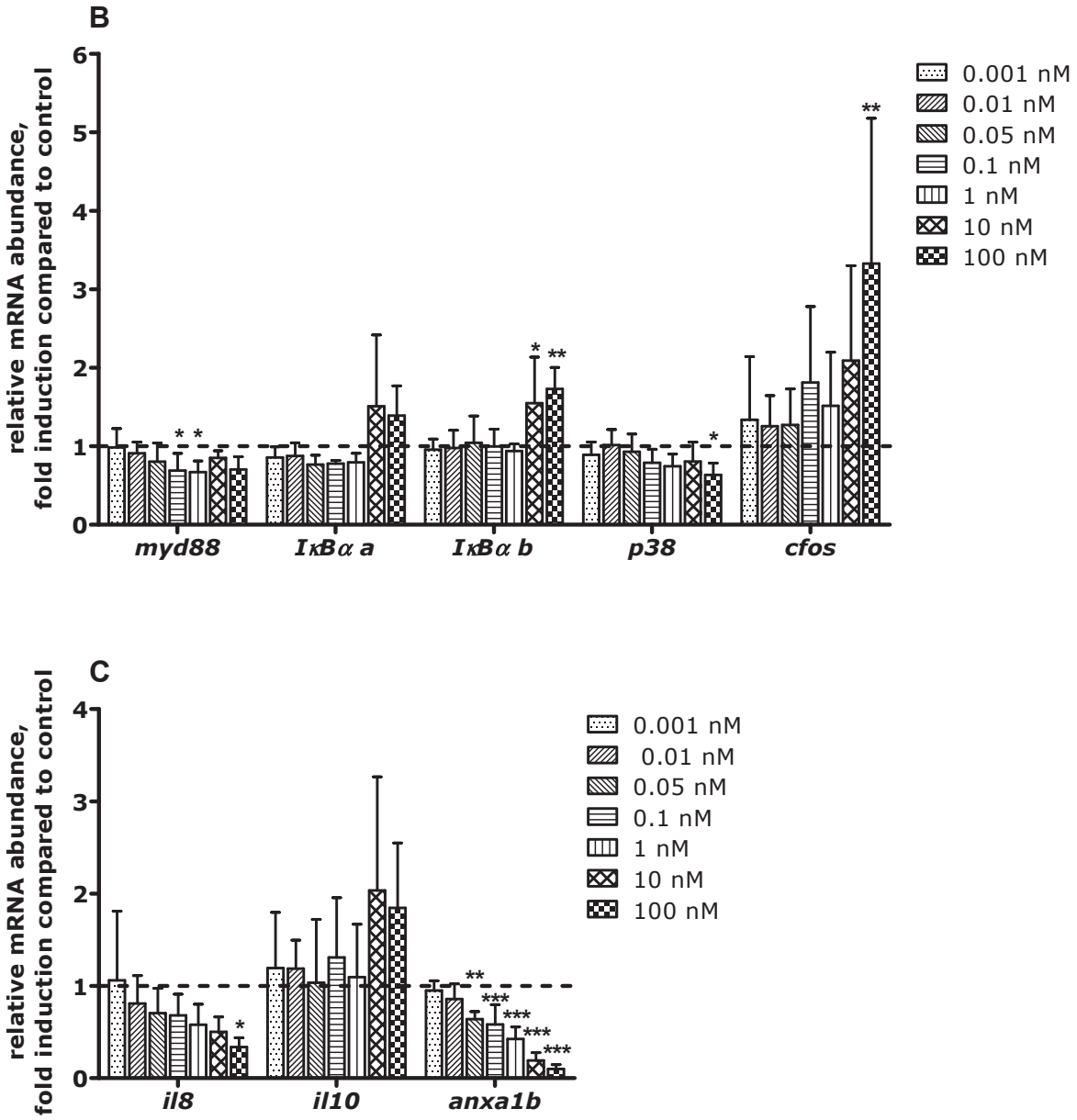


Figure 3.4: Relative mRNA abundance of inflammation-related genes after CP exposure. *Embryos were exposed to 0.001, 0.01, 0.05, 0.1, 1, 10 and 100 nM CP until 4 days post fertilization. At the end of the experiment, embryos were snap frozen in liquid N_2 and the expression of genes was analyzed by quantitative RT-PCR. Results are plotted as mean ± SD of fold changes compared to control. The control (fold change=1) is marked by a dashed line. Nominal CP exposure concentrations are shown. A: genes playing a role in cellular defense, inflammation and cell cycle regulation; B: members of the MyD88-dependent pathway; C: inflammatory mediators and an arachidonic acid synthesis inhibitor. N=5 independent experiments, except for 0.001, 0.05 and 10 nM groups, where N=4. The results were analyzed by one-way ANOVA followed by Dunnett's test, * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$.*

3.4 Discussion

We examined the uptake and potential immunosuppressive effects of a highly potent GC, CP, at environmentally relevant, nanomolar concentrations. Zebrafish embryos were continuously exposed from 0 to 4 dpf for all the experiments presented.

The uptake of CP was investigated in order to determine how much of this model GC can be taken up by zebrafish embryos. We found that organism-internal CP levels were quantifiable from ≥10 nM external exposure concentrations. Clobetasol, a potential metabolite of CP, was not detected in the embryo or the exposure medium. The additional propionate group of CP is at the C17 position on the molecule. The results of *in silico* molecular docking and molecular dynamics simulation experiments showed that substitutions at the C17 position of the cortisol backbone provides a more stable binding to both the human and zebrafish GRs (Sengupta et al., 2012). Thus, our results suggest that CP is not metabolized into clobetasol in zebrafish embryos. The amount of CP taken up by the embryos shows a linear correlation with the exposure concentrations. The BCF range calculated in this study is between 16 and 33. The estimated BCF of CP calculated by the software BCFBAF (v3.01, US EPA) is 95.5. Nakayama et al attempted to calculate a BCF of CP in adult common carp after exposure to 1 µg/L for 21 days. Since they did not detect any CP in the fish, they estimated a BCF of <16 (Nakayama et al., 2014). The LOQ value for the CP uptake measurement was 17 ng/g wet weight in the study by Nakayama et al, compared to our LOQ value of 5 ng/g wet weight. Furthermore, their sample size (on average one fish weighed 10.4 g wet weight) was much higher compared to ours (on average 28 embryos, total of 10.9 mg wet weight). Also, we already detected a quantifiable amount of CP after exposure to only 10 nM for 4 days. Thus, our method to measure CP in fish is more sensitive, and as we did detect CP, we were able to quantitatively derive a BCF.

The average daily dose of GCs in human treatments varies from 100 µg to 500 mg, depending on the compound preparation, and the route of administration (BNF, 2006). Thus, therapeutic concentrations also vary depending on these parameters. For instance, the plasma concentrations of CP were found to be between 100 and 450 ng/L in healthy subjects, after applying one fingertip-unit of a 0.05 % CP gel 3 times daily for one week on oral mucosa (Varoni et al., 2012). In another study, fluticasone propionate (FP), another highly potent GC, was investigated for signs of suppression of the HPA axis after inhalation of 1000 µg FP daily for one week. It was found that the basal urinary cortisol levels were significantly reduced after the measured maximum of 383 ng/L FP concentration in plasma, indicating the aforementioned suppression of the HPA axis (Brutsche et al., 2000). FP is a more potent GC than CP, based on their REPs in the GR-CALUX® assay (FP_{REP}=57, CP_{REP}=38; Macikova et al., 2014). Thus, the equivalent CP plasma concentration able to

suppress the stress axis would be 575 ng/L in humans. The fish plasma model (FPM) can be used to compare human therapeutic concentrations (H_TPC) with an estimated fish steady state plasma concentration (FPC_{ss}) (Fitzsimmons et al., 2001; Huggett et al., 2003; Macikova et al., 2014; Rand-Weaver et al., 2013). The predicted partitioning coefficient water : plasma ($P_{water:plasma}$) of CP is 47 (based on $LogK_{ow}$) and 106 (based on $LogD_{7.4}$). A minimal environmental water concentration that can cause harmful effects on fish can be calculated by assuming an effect ratio (ER) of 1, where ER equals to H_TPC/FPC_{ss}. By using the calculated 575 ng/L as the H_TPC, 5.4 ng/L (0.01 nM) based on $LogD_{7.4}$, or 12.2 ng/L (0.03 nM) based on $LogK_{ow}$, can cause GR-mediated effects. The plasma concentrations of another GC, beclomethasone dipropionate (BDP), the prodrug, and beclomethasone 17-monopropionate (17-BMP), the active molecule, were measured in adult fathead minnows in a 21-day exposure experiment (Margiotta-Casaluci et al., 2016). It was found that the measured average $P_{water:plasma}$ after exposure to 10 ng/L was 150, and after exposure to 1000 ng/L BDP was 54, while the predicted values were 124 and 65, based on $LogD_{7.4}$ and $LogK_{ow}$, respectively. Thus, the FPM prediction seems to be in good agreement with the measured values. When we used our calculated BCF values of 16 and 33, this internal concentration in the whole embryo would occur after external exposure to 17-36 ng/L (0.04-0.08 nM) CP. The results of the FPM model and calculations based on the quantified BCF are in good agreement. Thus effects of CP on the HPI axis of fish are expected to occur after exposure to environmental water concentrations between 0.01 and 0.08 nM (5.4-36 ng/L).

As GCs have immunosuppressive effects in mammals, we were interested whether the same effect can be seen in zebrafish embryos. The effects of CP on the innate immune response in zebrafish embryos were examined by the LPS challenge assay. LPS triggers inflammation due to the release of inflammatory mediators, such as the cytokine interleukin 1 β (il1β), the chemokine il8, prostaglandins, leukotrienes and reactive oxygen species (ROS) and/or nitric oxide (NO) resulting from e. g. oxidative stress (Di Rosa et al., 1990; Fujishima et al., 1993; Harizi et al., 2002; Hsu and Wen, 2002). Using a high concentration of LPS leads to the overproduction of these mediators, resulting in death. Thus, elevated survival rates would indicate immunosuppression in CP-exposed embryos. LPS challenge assays were carried out with CP-exposed embryos with and without 0.001 % DMSO used as solvent vehicle. Apart from the LPS challenge assay, the respiratory burst of the zebrafish embryos exposed to 0.001% DMSO, or 1, 10, 100, 1000 nM CP was also examined. The respiratory burst assay (RBA) measures the amount of ROS produced by macrophages after induction (Hermann *et al.*, 2004). The respiratory burst was significantly decreased after exposure to 1000 nM CP with DMSO (**SI Fig. 3.2**).

The 0.001% DMSO content is 10 times lower than the DMSO concentration accepted for use in zebrafish embryo experiments to assess fish acute toxicity (OECD guideline 236). In the

RBA assay, DMSO as a solvent vehicle did not cause differences between the vehicle and blank controls. Our results suggest that exposure to 1 μM CP either impaired the ability of macrophages to produce ROS, or decreased the number of macrophages in the embryos. This is an indication of immunosuppression. However, when DMSO was used as a solvent carrier in the LPS challenge assay, it masked the immunosuppressive effect of CP, as CP-exposed embryos showed a significantly lower survival rate compared to the blank control. As DMSO is a well-known permeability enhancing agent (reviewed in Yu and Quinn, 1998), our results are likely due to the fact that the embryos had taken up higher amounts of LPS when DMSO was present in the system, and thus the immune system was reacting faster and stronger. The concentration of LPS used in this assay is a critical parameter, since zebrafish embryos react sensitively to slight changes. However, the same survival rates of the DMSO control embryos and the blank control embryos in the experiments without DMSO – where higher LPS concentration was used in order to see the lower mortality in the CP-exposed groups – indicate that the same amount of LPS was taken up by these groups, which are used then to evaluate the CP effects on the embryos. The results from experiments conducted with DMSO vehicle are reported here in order to draw attention to the fact that solvents can have an effect on the biological model and/or assay used. If we had performed the LPS challenge assay only with DMSO and compared the results only to that group, we would have concluded incorrectly that CP caused no immunosuppression in zebrafish embryos up to 1 μM concentration in the LPS challenge assay. Most of the studies investigating the effects of GCs (and other potentially toxic substances) on fish (and other models too) use a solvent to dissolve GCs, in many cases DMSO. Our suggestion is to avoid the use of solvent carriers in the experiments, if possible. In cases where the use of a vehicle cannot be avoided, the testing of negative controls is essential, in order to determine whether the solvent has an effect on the endpoints used to observe the specific effects the study focuses on. If there is a solvent effect, more concentrations of the solvent should be tested to analyze the results as a mixture of the solvent and the compound tested (Stevens et al., 2014). Alternatively, passive dosing would be an option to circumvent the solvent vehicle (Gilbert et al., 2015).

In experiments carried out without DMSO, CP showed a strong and concentration-dependent effect on the LPS-induced inflammatory responses of the embryos. The survival rate of exposed embryos was higher than for the blank controls, indicating immunosuppression. This effect was significant already at 0.1 nM, which is close to environmentally relevant concentrations. As described earlier, the impairment of immune response after GC exposure has previously been observed in fish. However, the exposure concentrations used in many previous studies (for example Gadan et al., 2012; Nakayama et al., 2016; Sharif et al., 2015) were much higher than the environmentally relevant ng/L concentrations, except for the one

study where a significant decrease in lymphocyte number of adult fathead minnows after exposure to 10 ng/L (0.02 nM) BDP for 21 days was found (Margiotta-Casaluci et al., 2016). Among lymphocytes, natural killer cells belong to the innate immune system attacking viruses, while B and T cells belong to the adaptive immune system. The REP of BDP is 0.53, but its active metabolite, 17-BMP, has a REP value of 13. Therefore, 17-BMP is three times less potent to activate the GR than CP. On the other hand, the applied endpoint is characteristic to the adaptive immune system too, which is not yet present in zebrafish embryos. Hence, the results of the two tests are difficult to compare. Thus, to our knowledge, our study is the first to demonstrate a strong suppression of innate immune system in fish already at 0.1 nM GC, an environmentally relevant concentration. Furthermore, our results support the previously described estimation of the effective concentration of CP to be between 0.01-0.08 nM.

The transcription of inflammatory response-related genes was investigated in this study to reveal the molecular pathways behind the strong immunosuppressive effect of CP. Among the investigated genes, *hsp70* and *nrf2* are connected to cellular defense mechanisms, while *p53* plays a role in inflammation and cell cycle regulation. *Nrf2* and *p53* were not regulated, but *hsp70* was significantly up-regulated after CP exposure in zebrafish embryos. *Hsp70* is part of the cytosolic GR complex (Hutchison et al., 1994), and was shown to regulate GR ligand-binding by inactivating the GR when bound to it (Kirschke et al., 2014). Thus, the up-regulation of *hsp70* can also be linked to the stress response itself. Consequently, the up-regulation may mean a negative feedback responding to the constant GR-activation by CP. Furthermore, excess *hsp70* expression was shown to suppress the immune system in rats by preventing LPS-induced cytokine production (Dokladny et al., 2010).

As already mentioned, the inflammatory mediators released upon induction by LPS are regulated by the MyD88-dependent pathway. Thus, we examined some of the key participants in these cascades. The slight down-regulation of *myd88* itself may cause the down-regulation of the signaling via the TLRs. Other elements of the cascade showed stronger regulation upon CP exposure. The increased *IκBα* transcripts indicate the inhibition of the NFκB pro-inflammatory pathway (Jacobs and Harrison, 1998; Verma et al., 1995). *IκBα* was up-regulated in zebrafish embryos after exposure to 100 μM dexamethasone, consistent with our results, but at a 1000x higher concentration (Schaaf et al., 2009). Interestingly, the aforementioned role of *hsp70* in preventing LPS-induced cytokine production was associated with the inhibition of *IκBα* degradation, and thus repressing the NFκB pro-inflammatory pathway (Dokladny et al., 2010). Our results support this connection, as the gene expression profiles of *hsp70* and *IκBα* were very similar in our experiments. Thus, this phenomenon seems to be conserved from mammals to fish. The down-regulation of the member of the MAPK-signaling pathway *p38* is also in agreement with the results

observed in the LPS challenge assay. Dexamethasone was shown to inhibit MAPK phosphatase 1 and thus down-regulate the MAPK pathway in mouse macrophages (Abraham et al., 2006). This pathway also phosphorylates and thus activates the transcription factor AP-1 (Guha and Mackman, 2001), which has C-Fos and C-Jun subunits. *Cfos* showed the highest upregulation after CP-exposure. *Cfos* expression is also used as an indirect marker of neuronal activity (Dragunow and Faull, 1989). LPS-induced *cfos* induction seems also to be exaggerated in the brains of HPA-axis deficient rats, indicating higher neuronal activity (Conde et al., 1999). Due to the different roles of C-Fos, its transcriptional up-regulation indicates an increase of AP-1, but it may also mean an elevated neuronal activity. This would need further investigation.

The significant dose-dependent down-regulation of the pro-inflammatory chemokine *il8* and the trend of up-regulation of the anti-inflammatory cytokine *il10* are also in line with the observed lower mortality of the embryos after LPS challenge. The serum levels of il10 have been shown to increase after GC treatment in humans (Stelmach et al., 2002), while *il8* was down-regulated after exposure to 100 µM dexamethasone in zebrafish embryos (Schaaf et al., 2009). As already mentioned, however, this concentration is 1000x higher than that used in our study. Injury-induced *il8* was also down-regulated after 250 nM beclomethasone exposure in zebrafish hearts (Huang et al., 2013). To our knowledge our study is the first to show significant down-regulation of *il8* after exposure to 100 nM GC. Furthermore, the p38 MAPK pathway was shown to be involved in the production of *il8* after LPS stimulation in equine leukocytes (Neuder et al., 2009). In agreement with this finding, both of these transcripts were decreased in our experiments, both of them showing statistical significance after exposure to 100 nM CP. This highlights yet another part in the LPS-induced inflammation cascade that is conserved between mammals and fish.

Anxa1b showed the most striking effect, as it was strongly down-regulated in a concentration-dependent way. The effect was significant at 0.05 nM CP (23.3 ng/L), that is within the environmentally relevant range of GCs. The anti-inflammatory role of ANXA1 in mammals is due to the inhibition of eicosanoid synthesis by suppressing phospholipase A2. It was also shown to inhibit the cytokine production induced by LPS in rodents and human cells (Girol et al., 2013; Yang et al., 2009). *Anxa1b* was also significantly down-regulated after exposure to 1 µM GCs (beclomethasone, beclomethasone dipropionate, dexamethasone and hydrocortisone) in zebrafish embryos following the amputation of their caudal fins (Sengupta et al., 2012). Another regeneration experiment focusing on cardiac repair in zebrafish showed the up-regulation of this gene in the hearts after 250 nM beclomethasone exposure (Huang et al., 2013). These controversial results in fish can be due to tissue specificity of *anxa1b* response to GCs. The effect of 0.05 nM CP in the LPS challenge assay was not measured; 0.01 nM did not cause significant immunosuppression

yet, but 0.1 nM did. As *anxa1b* appears to have a trend of significant down-regulation that closely follows the observed higher survival in the LPS assay, it may be an indication of the role of this gene in the observed immunosuppressive effect of CP. However, the effect seems to be the exact opposite of what was found in mammals. If *anxa1b* inhibits LPS-related cytokine production, then the embryos should have died faster due to the excess amount of cytokines resulting from the down-regulation of this gene. Hence, it is also possible that another important factor in the inflammatory process, not examined in this study, counteracts the down-regulation of *anxa1b.* It is moreover noteworthy that there are three variants of *anxa1* genes in zebrafish: *anxa1a, -b and –c*, resulting from genome duplications (Farber et al., 2003). Thus, *anxa1b* may have another function and be regulated by GCs in a different manner than in mammals. Regardless of the function, the strong response of zebrafish embryo *anxa1b* to low nanomolar concentrations of GCs may be used to monitor GCs in the environment. This potential application needs further investigation and validation.

3.5 Conclusions

The main goal of this study was to assess whether CP at environmentally relevant concentrations is taken up and causing immunosuppression in zebrafish embryos. We could show that CP is indeed taken up by the embryos, and it is not metabolized to another GC, clobetasol, a potential metabolite of CP. Therefore, CP appears to be the active drug acting in the zebrafish embryos. The BCF of CP (16-33) was calculated for the first time based on measured concentrations. Zebrafish embryos were challenged by LPS in order to examine the effects of CP on the inflammatory response. DMSO as a solvent carrier caused significant effects compared to the negative control, and it also masked the effect of CP in the LPS assay. Without using a solvent carrier, we were able to show that CP did cause a significant suppression of the innate immune response of embryos already after exposing them to a nominal concentration of 0.1 nM CP (46.7 ng/L), that is close to the detected concentrations in environmental water samples, and also close to the effective concentration predicted by FPM and calculations based on BCF (0.01-0.08 nM). The gene expression analyses of various inflammation-related genes mostly supported the observed results in the LPS challenge assay: the MAPK signaling (*p38, il8*) and NFκB pro-inflammatory (*IκBα)* pathways were down-regulated, while the general stress and the cytosolic GR complex component (*hsp70*) was up-regulated after CP exposure. *Anxa1b*, a GR-target immune function-related gene was significantly down-regulated at already 0.05 nM, which is within the predicted effective CP concentration range. Thus, *anxa1b* is a promising candidate gene that can be further evaluated for its potential to be used for monitoring GR-active compounds in the environment. Monitoring water pollutants with zebrafish embryos has the advantage of using the whole organism. This is superior to the established GR-CALUX® *in vitro* test, which can only detect compounds activating the GR, while zebrafish embryos have the potential to detect compounds able to interfere with the HPI axis at any level.

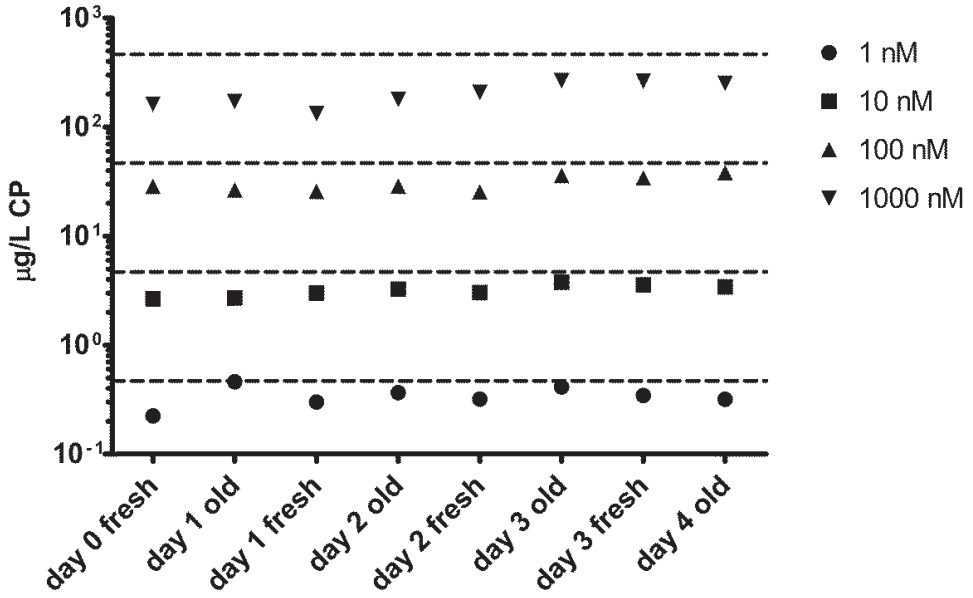
This study showed for the first time that a model GC present in the environment suppresses the innate immune system of fish at environmentally relevant concentrations. This may make fish more susceptible to pathogens, reducing their survival chances. Thus, GCs should be monitored in the aquatic environment, and their influence on the sensitivity of fish to various pathogens and other environmental stressors be explored.

Contributions: I conducted all the zebrafish embryo exposures, LPS challenge assays, and qRT-PCR analyses. I also designed 3 of the applied primers. I carried out the LC-MS/MS analyses with the assistance of René Schönenberger, and I also evaluated all the data demonstrated in this chapter.

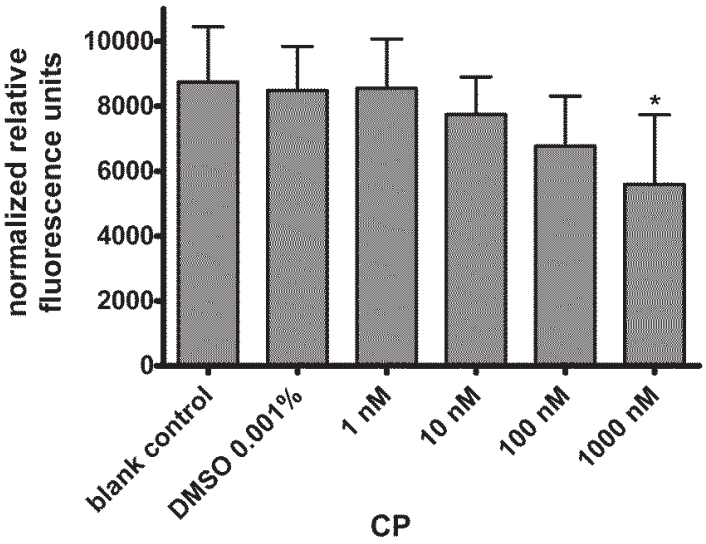
3.6 Supplementary information

SI Table 3.1: Overview of primers used in this study for qPCR. *F and R stand for forward and reverse directions.*

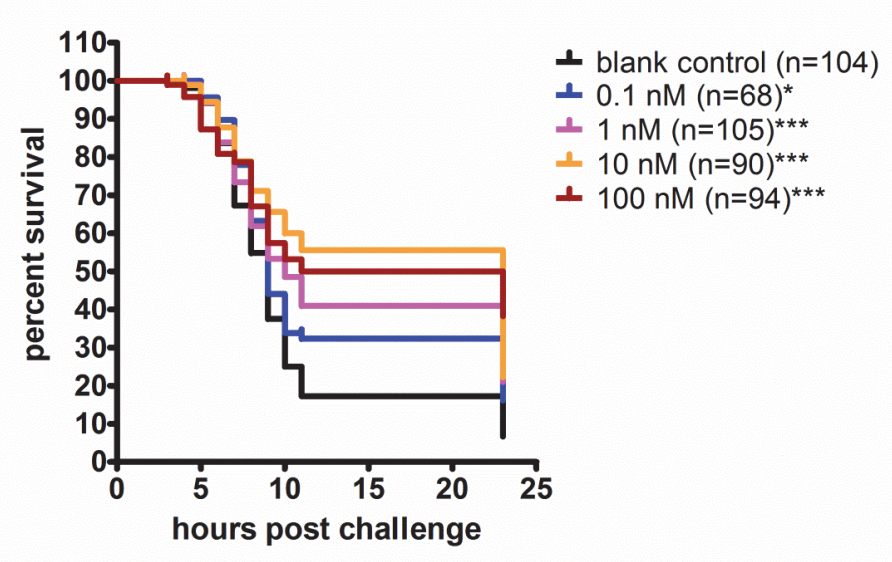
Gene	Name	Biological function	Direction	Sequence (5' → 3')	Acession number	primer efficiency	Reference
annexin A1b, lipocortin 1	*anxa1b*	phospholipase A2 inhibitor (eicosanoid synthesis)	F	AAACTACCCTGCTTGCCCTTT	NM_181759.1	1.86	this study
			R	GATGCTCTCCTGCCTCGTAT			
chemokine (C-X-C motif) ligand 8a (interleukin 8)	*il8*	inflammatory chemokine	F	AAGCCGACGCATTGGAAAAC	XM_001342570.5	1.90	Mottaz et al., under review
			R	GTTGTCATCAAGGTGGCAATGA			
nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha a	*IκBα a*	inhibition of NFκB	F	CAGCAAGCCTGAAGGAGACA	NM_213184.1	1.95	this study
			R	GAGCAGTCTGTCTCTGGTTGT			
nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha b	*IκBα b*	inhibition of NFκB	F	TTTGGACAGCGGGTTGGATT	NM_199629.1	1.95	this study
			R	AGTGCAGTCTGTCTCTGGTTG			
v-fos FBJ murine osteosarcoma viral oncogene homolog Ab	*cfos*	constituent of AP-1 (MAPK pathway)	F	CCGCACACAAACCCATCTG	NM_205569.1	1.95	Jin et al., 2010
			R	GGTCGTGATTGATGTGTTTGGT			
myeloid differentiation primary response 88	*myd88*	adaptor protein of TLR4, regulating the MyD88-dependent pathway	F	TCCGAAAGAAACTGGGTCTG	NM_212814.2	1.98	Hsieh et al., 2010
			R	TCGTCGTCTAGAATTTCTTTGAGC			
heat shock cognate 70-kd protein, tandem duplicate 1	*hsp70*	cellular defense, part of the cytosolic GR complex	F	CAACAACCTGCTGGGCAAA	NM_131397.3	1.84	Keegan et al., 2002
			R	GCGTCGATGTCGAAGGTCA			
mitogen-activated protein kinase 14a	*p38*	p38 MAPK	F	AACGTGACGGTGGACATTTG	NM_131722.1	1.98	Sheng et al., 2016
			R	TGGGCATCTGAGGAAGTGAG			
interleukin 10	*il10*	inflammatory cytokine	F	GCTCCGTTCTGCATACAAAGA	NM_001020785.2	1.63	Mottaz et al., under review
			R	TGGGGTTGTGGAGTGCTTTA			
nuclear factor, erythroid 2-like 2a	*nrf2*	cellular defence	F	GACAAAATCGGCGACAAAAT	NM_182889.1	1.89	Shi and Zhou, 2010
			R	TTAGGCCATGTCCACACGTA			
tumor protein p53	*p53*	cell cycle regulation, inflammation	F	AAGAACAGCCTCAGCCATCC	NM_001271820.1	1.97	He et al., 2014
			R	CAGGTCCGGTGAATAAGTGC			
eukaryotic translation elongation factor 1 alpha 1	*ef1α*	housekeeping gene, used for normalization	F	GGAGTGATCTCTCAATCTTGAAACT	NM_131263.1	1.86	Mottaz et al., under review
			R	TGTGGGTCTTTTCCTTTCCCA			



SI Figure 3.1: Measured exposure media concentrations during one of the three uptake experiments. *500 µL samples from the exposure media was taken out from the petri dishes right before ("old") and right after ("fresh") changing the solution into glass autosampler vials during one of the three uptake exposures. The samples were stored at -20 ºC until analysis. The exposure concentrations at each time point were determined by the same LC-MS/MS method as for the embryo extracts. The nominal exposure concentrations are demonstrated as dashed lines on the graph.*



SI Figure 3.2: Influence of clobetasol propionate with DMSO as a solvent vehicle on the respiratory burst of zebrafish embryo macrophages. *The embryos were exposed to 0.001 % DMSO or 1, 10, 100 or 1000 nM CP in DMSO in small petri dishes until 5 days post fertilization. The respiratory burst assay was carried out as described in Hermann et al., 2004. Briefly, the embryos were plated onto 96-well plates with 100 µL exposure solution on day 4 in the afternoon. On day 5, early morning 100 µL reaction mix with or without 400 ng/mL phorbol myristate acetate (PMA) was added to each well. PMA induces the macrophages to produce reactive oxygen species (ROS) by stimulating protein kinase C to phosphorylate and thus activate NADPH oxidase to produce ROS. The reaction mix contained 1 µg/mL 2',7' – dihydrodichlorofluorescein diacetate (H_2DCFDA) and 0.2 % DMSO. The nascent ROS oxidates the non-fluorescent H_2DCFDA into the fluorescent dichlorofluorescein (DCF). After the addition of the reaction mixes, fluorescence was monitored with a TECAN infinite M200 spectrophotometer (TECAN, Männedorf, Switzerland) for 4 h 15 min with 3 min intervals in order to plot the kinetic curve of ROS production. The parameters of the measurement were: excitation: 485 nm, emission: 530 nm, number of flashes: 25, integration time: 20 µs. In each experiment, 6 embryos/exposure group were not induced, and 6 embryos/exposure group were induced by PMA in order to normalize with the fluorescence values from the non-induced embryos. The kinetic curves of each experiment are not demonstrated here. The graph represents the relative fluorescence units at 240 min (that is close to the plateu of the kinetic curve) averaged per exposure group and normalized with the non-induced average values. N=5 independent experiments, mean±SD, *p<0.05, one-way ANOVA followed by Dunnett's test.*



SI Figure 3.3: Influence of clobetasol propionate without DMSO vehicle on the survival of zebrafish embryos with a different genetic background after bacterial lipopolysaccharide challenge. *The embryos used in these experiments had genetic backgrounds mixed from WiK and OBI zebrafish. The embryos were exposed to 0.01, 0.1, 1, 10, 100 nM CP dissolved directly in the dilution water until 4 days post fertilization, then challenged with LPS at 97 hours post fertilization, and the survival rate was monitored from 2 to 11 hours post challenge hourly (hpc), and at 23 hpc. The results are plotted as Kaplan-Meier survival curves (N=4 independent experiments, total number of embryos (n) per concentration point indicated on the graph; *p<0.05, *** p<0.0005, Log-rank test compared to blank control).*

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Chapter 4:

Biomarker search and validation for environmental synthetic glucocorticoid exposure and/or anti-inflammatory pathways in zebrafish (*Danio rerio*) embryos

Synthetic glucocorticoids (GCs) are human and veterinary pharmaceuticals that mimic the natural stress hormone cortisol and are used for their anti-inflammatory effects. GCs bind and activate the glucocorticoid receptor (GR) and thus regulate gene expression patterns of e.g. development, immune response, osmoregulation and glucose metabolism. They can reach the aquatic environment due to incomplete elimination in wastewater treatment plants (WWTPs). As demonstrated in Chapter 3 of this thesis, anti-inflammatory effects have been observed in fish after exposure to environmentally relevant concentrations. The currently available reporter gene assays used for assessing the total GR activity of environmental water samples, such as the GR-CALUX®, are only providing information on whether the compounds present in the sample can activate the GR, but nothing on other effects along the fish stress response axis. Thus, our goal was to find candidate biomarkers of GC exposure and effect on the mRNA and/or protein level in the zebrafish (*Danio rerio*) embryo model. In the previous study (Chapter 3 of this thesis) we found several inflammatory response-related genes that were regulated on the mRNA level after exposure to the highly potent GC clobetasol propionate (CP). In order to see whether the observed effects on these genes are GC-specific, we here exposed the zebrafish embryos to the non-steroidal anti-inflammatory drug (NSAID) diclofenac (DCF). Moreover, we used environmental grab water samples in which GCs and DCF had been detected to determine whether the observed effects are robust enough to be detected even in a complex environmental matrix. After DCF and environmental water exposures, the candidate biomarker genes were quantified with real-time PCR for mRNA transcript abundance. Furthermore, we developed an LC-MS/MS based targeted proteomics method that is able to simultaneously measure 12 GC-action related genes in zebrafish embryo digests. This method was used on CP-, DCF- and environmental water exposed-embryos. Our results show that *cfos* (infolved in anti-inflammatory action and neural activity) and *anxa1b* (key regulator of the arachidonic acid synthesis) transcripts were regulated by both steroidal and non-steroidal anti-inflammatory drugs, and the effects were robust enough to be detected after exposure to the complex environmental water samples. However, *cfos* has multiple roles; for example, it has been associated with neuronal activity as well. In comparison, *anxa1b* is a specific potential biomarker to detect compounds that can cause anti-inflammatory effects in zebrafish embryos. The targeted proteomics analysis revealed that the muscle protein Myhz2 is

regulated after NSAID, but not after CP exposure. IκBα that inhibits the NFκB pro-inflammatory pathway was shown to be regulated by GCs only on both the transcript and protein levels. However, the response of these two proteins was masked by the complex matrix of the grab water samples. Thus, we propose to further investigate whether *anxa1b* can be used to develop a bioassay for the detection of anti-inflammatory activity in the aquatic environment.

Keywords: anti-inflammatory pharmaceuticals, teleost fish, mRNA expression, targeted proteomics, Anxa1b, IκBα, Il8, Cfos, Myhz2, Nhe3b

4.1 Introduction

Synthetic glucocorticoids (GCs) are potent anti-inflammatory drugs widely used in human and veterinary medicine. They act by mimicking cortisol, the natural stress hormone in humans and fish and hence are potential endocrine disruptors, interfering with the stress axis, that is the hypothalamus-pituitary-interrenal (HPI) axis in fish, and the hypothalamus-pituitary-adrenal (HPA) axis in mammals. GCs have been detected in the environment in the low ng/L concentration range (Ammann et al., 2014; Herrero et al., 2014; Jia et al., 2016; Liu et al., 2015; Macikova et al., 2014; Nakayama et al., 2016). As cortisol mimics, they activate the glucocorticoid receptor (GR) and thus can cause diverse harmful effects on non-target organisms, such as impairment of glucose metabolism (Kugathas and Sumpter, 2011), bone formation (Hillegass et al., 2008), osmoregulation (Lin et al., 2011), and immune function (Kugathas et al., 2013). Recently it was found that the lymphocyte count of fathead minnow decreased after 10 ng/L beclomethasone dipropionate exposure (Margiotta-Casaluci et al., 2016). Furthermore, a highly potent, environmentally relevant GC, clobetasol propionate (CP), has been shown to significantly decrease the inflammatory response in zebrafish embryos after exposure to >45 ng/L (Hidasi et al., under review; see Chapter 3). These recent findings prove that GCs present in the aquatic environment suppress the immune system of fish and thus impair their defense against pathogens.

During a European monitoring project, the GR-CALUX® *in vitro* bioassay was used to detect GR-active environmental water samples (Van der Linden et al., 2008). Activities were found in four catchments, including the Emme catchment in Switzerland (Tousova et al., in preparation). The GR-active Swiss site was downstream of a wastewater treatment plant (WWTP), and diclofenac (DCF) was detected there as well. In a follow-up study at that site, GR activity was further investigated with effect-directed analysis (EDA), and CP and fluticasone propionate were identified as the compounds responsible for the biological effect (Sonavane et al., in preparation). EDA is a method that combines bioassays and analytical chemistry in order to identify environmental pollutants causing biological effects (Brack, 2003).

This bioassay is a human cell line-based reporter gene assay that identifies compounds that are able to interact with the human GR. However, it does not tell us whether these compounds would also interfere with the fish GR, and it does not detect compounds that interfere with the HPI axis at other levels than GR interaction. This situation creates the necessity to find biomarkers in fish that are indicative of compounds able to interfere with the fish stress axis on all levels. The zebrafish is not only an ideal model for GC research (Alsop and Vijayan, 2009; Schaaf et al., 2009), but can also be used for deciphering molecular mechanisms behind the effects of environmental pollutants using various tools that target the

mRNA and protein levels (Groh et al., 2011; Groh et al., 2013). Analyzing gene expression on the mRNA and/or protein levels after exposure to a model GC such as CP, can identify candidate biomarkers. However, even though gene expression on the mRNA level is informative, it only reflects the initial step potentially leading to functional proteins. Thus, measurements on the protein level give a more integrated understanding of the molecular response mechanisms activated after exposure to single compounds and/or environmental water samples. Selected Reaction Monitoring (SRM) is a liquid chromatography-tandem mass spectrometry (LC-MS/MS) quantitative proteomics technique (Lange et al., 2008; Picotti and Aebersold, 2012) that has become popular for studying protein expression in zebrafish. For example, SRM has been used for studying the sexual differentiation process in zebrafish gonads (Groh et al., 2013).

Potential biomarkers need to be specific to the mode-of-action of interest. To test specificity, other compound(s) affecting the same pathway, but not via the same mode-of-action (see **Fig. 4.1**) can be used for validation. In many cases, this is difficult to achieve because there is crosstalk between molecular pathways. Potential biomarkers need moreover to react significantly after exposure to environmentally relevant concentrations in order to avoid sample enrichment. This is because enrichment can introduce artifacts and may necessitate the use of a solvent, which may interfere with the results (see Chapter 3; Oliveira et al., 2016). Finally, biomarkers need to be robust, i.e. unaffected by a complex environmental matrix, which could influence speciation or cause ion suppression.

GCs are not the only class of anti-inflammatory drugs. Non-steroidal anti-inflammatory drugs (NSAIDs) are a group of drugs that inhibit cyclooxygenase (COX)-1 and/or -2, and hence the production of prostaglandins and thromboxanes. This cascade is part of the arachidonic acid pathway that is also inhibited by GCs. Next to aspirin, diclofenac (DCF) is one of the most frequently used NSAIDs. **Fig. 4.1** compares the mode-of-action of GCs and DCF in a schematic way. DCF has been detected in the aquatic environment in the range of ng/L to low µg/L concentrations (Moschet et al., 2013; Schroder et al., 2016; Tousova et al., in preparation). Furthermore, DCF has been added to the first watch list of priority hazardous substances in the EU in 2013. A computational study using molecular docking showed that the zebrafish enzyme COX-2 can bind DCF (Walker and McEldowney, 2013).

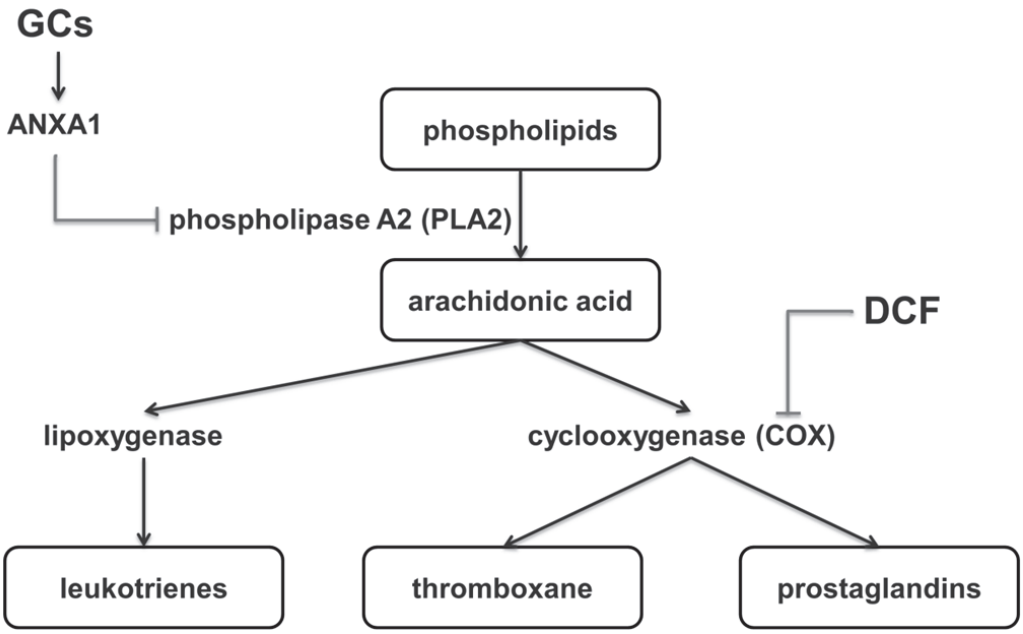


Figure 4.1: Schematic representation of synthetic glucocorticoid (GC) and diclofenac (DCF) mode-of-actions in mammals.

The main goal of this study was to identify biomarkers of GC and/or anti-inflammatory action after exposure to the model GC, CP, and the NSAID DCF. For this purpose, a group of inflammatory genes already shown to be regulated on the mRNA level after CP exposure (Chapter 3) were examined on the mRNA level after DCF exposure of zebrafish embryos. Moreover, a SRM targeted proteomics method was developed to monitor GC-action related proteins after CP and DCF exposure. We collected grab water samples from the Swiss site described above, up- and downstream of the WWTP and from its effluent. These samples were analyzed for the presence of GCs and DCF, and were used for exposures in order to see whether the potential mRNA or protein biomarkers have an effect robust enough to be seen even in a complex matrix.

4.2 Materials and methods

4.2.1 Chemicals

Clobetasol propionate (CP), diclofenac sodium salt (DCF), formic acid for MS (FA), ammonium formate standards for the chemistry method (listed in Ammann et al., 2014), and the salts used to prepare the dilution water (294 mg/L $CaCl_2 \cdot 2\ H_2O$, 123.2 mg/L $MgSO_4 \cdot 7\ H_2O$, 64.74 mg/L $NaHCO_3$, and 5.75 mg/L KCl; prepared according to OECD test guideline 236) were purchased from Sigma-Aldrich (Switzerland). HPLC-gradient pure acetonitril (ACN) was purchased from Acros Organics (Chemie Brunschwig AG, Switzerland). LC-MS

grade methanol (MeOH) was from Optima (Fischer Scientific, Switzerland), and chloroform from Merck (Switzerland).

4.2.2 Environmental sample collection and preparation

The environmental water samples were collected from the river Urtenen upstream of the municipal WWTP in Kernenried, Switzerland, from its effluent and downstream on March 30th, 2016. We sampled at the time when the morning bathroom peak is expected to be discharged to the river. The hydraulic retention time of the WWTP is approximately 36 hours based on the information obtained from the WWTP, thus we collected our samples between 5 and 6 in the afternoon. Approximately 8 L water per site was collected into aluminum bottles. Before sample collection, temperature, O_2 content, conductivity and pH of the river were measured using a multimeter (Multi 3430 SET G, WTW, Germany) (**SI Table 4.1**). After collection, the samples were transported back to the lab and were stored overnight at +4 ^{0}C in the dark. Next day, the samples were filtered through 0.2 µm pore-sized filters (Sartorius). 2L sample were filtered for zebrafish embryo exposures, and then stored again at +4 ^{0}C in the dark. For the chemical analysis, one L OECD dilution water was prepared to be used as the negative control sample. One L of negative blank and water samples spiked with internal standards were filtered the same way. Then, the sample preparation for chemical analysis was carried out according to the protocol described in (Ammann et al., 2014). Briefly, 15 mM ammonium formate was added to each sample, the pH measured and found to always be between 7.46 and 7.86. Next, samples were enriched by using mixed-bed solid phase extraction (SPE) cartridges. Each cartridge (Macherey Nagel AG Chromabond ready to use SPE cartridges) was filled with a mixture of sorbents containing 150 mg Isolute ENV$^+$ (Separtis), 100 mg Strata X CW (Sepra ZT-WCX), 100 mg Strata X AW (Sepra ZT-WAX) and 200 mg Oasis HLB. The cartridges were conditioned with a 1:1 mixture of 10 mL ethyl acetate (EtOAc) : methanol (MeOH), then with 10 mL MilliQ water. After extraction, the cartridges were dried under a gentle flow of N_2. Sequential elution was performed with 5 mL EtOAc:MeOH 1:1 with 1 % formic acid (FA) and 3 mL EtOAc:MeOH 1:1 into the same glass vial then with 5 mL EtOAc:MeOH 1:1 with 1 % NH_3 into a second glass vial. The acidic and basic fractions were evaporated separately under a gentle N_2 flow. The residues were reconstituted in 100 µL ACN:H_2O 1:1 mixture and stored at -20 ^{0}C until the chemical analysis.

4.2.3 Chemical analysis of environmental samples

The applied LC-MS/MS SRM method targeting GCs has been developed in our research group (Ammann et al., 2014). Briefly, 10 µL sample were injected using a PAL autosampler. LC separation was achieved on a Poroshell EC C18 column (100x2.1 mm, 1.7 µm, Agilent)

using a 26 min water/ACN gradient with 5 mM ammonium formate at 200 µL/min flowrate. Two SRM transitions per analyte were monitored during three time segments in positive mode electrospray (Vantage, ThermoFisher Scientific). The first transitions were used for quantitation and the second as qualifiers. Needle voltage was set to +3000 V, vaporizer temperature to 150 °C, capillary temperature to 250 °C. Collision energies were between 18 and 47 V, depending on the compound monitored. The concentrations were calculated from the area under the quantifier peaks using standard calibration curves. DCF was added to the list of target compounds using a collision energy of 30 V and the transitions m/z 296.1 → 249.9 / 214.0.

4.2.4 Zebrafish husbandry and embryo collection

Wild type embryos with mixed genetic background from WiK (Max Planck Institute for Developmental Biology, Tübingen, Germany), OBI (Helmholtz Centre for Environmental Research established from OBI hardware store, Leipzig, Germany) and Qualipet (petshop, Switzerland) were used in the experiments. Zebrafish were maintained according to recommended procedures (Nüsslein-Volhard and Dahm, 2002). Adult zebrafish were kept in a Mass Embryo Production System (Aquatic Habitats®, Pentair Aquatic Eco-Systems, USA) with a mix of tap and reverse-osmosis water (1:1) using a 14/10 h light/dark cycle at 28 °C. The embryo collection system was placed in the tank in the afternoon the day before collection. The embryos were collected 45 min after the light turned on in the morning, washed, and kept in dilution water (prepared according to OECD guideline 236). Next, the fertilized embryos were selected and placed in the pre-soaked big (d=94 mm) or small (d=60 mm) petri dishes, depending on the experiment type.

4.2.5 Zebrafish embryo exposures

For the CP exposures, approximately 0.5-1 mg CP was dissolved in one liter of dilution water and the concentration was measured by a UV-Vis spectrophotometer (Cary 100, Agilent Technologies, Switzerland) at λ=240 nm wavelength. The stock solution was kept at 4 °C up to one week. Embryos were exposed to 0.1, 1, 10 and 100 nM CP. For the DCF exposures, approximately 1-1.5 mg DCF was dissolved in one liter of dilution water and the concentration was measured by the UV-Vis spectrophotometer at λ=276 nm wavelength. Embryos were exposed to 0.1, 1 and 10 nM DCF. No dilution was applied for the water samples during exposures. Embryos were exposed in each experiment from 0 day post fertilization (dpf) to 4 dpf at 28 °C using a 14/10 h light/dark cycle. For qPCR analyses, 24 embryos in 12 mL were raised in small petri dishes. For the targeted proteomics experiments, 35-40 embryos in 40 mL were kept in big petri dishes. Before the experiments, big and small petri dishes were pre-soaked with the exposure solutions for one day. 8.5 mL

(small petri dishes) and 30 mL (big petri dishes) of the exposure solutions were changed every day during the experiments.

4.2.6 RNA isolation and quantitative real-time PCR (qPCR)

Zebrafish embryos were exposed to 0 (blank control, dilution water), 0.1, 1 and 10 nM DCF, or upstream, effluent and downstream water samples. The embryos were anaesthetized by ice-cold dilution water, collected and snap-frozen in liquid N_2 at 96 hours post fertilization (hpf). RNA was isolated using the Qiagen RNeasy Mini Kit according to the manufacturer's protocol (Qiagen AG, Switzerland). The isolated RNA concentration was measured by a Nanodrop® ND-1000 spectrophotometer (Nanodrop, USA). RNA was treated with DNase I (Amplification Grade, Invitrogen by Life Technologies) in order to remove possible genomic DNA contamination. 1 µg purified RNA was reverse transcribed to cDNA (PrimeScript™ RT Reagent Kit, Takara Bio Inc., Japan) in 20 µL volume using a temperature program starting with incubation at 37 °C for 15 min, then at 85 °C for 5 min, finally cooling down to 4 °C (Bio-Rad Laboratories DNA Engine Dyad™). In parallel, 1 µg RNA in RNase free water was ran without the reaction mix for negative controls. For the qPCR analyses, the reaction mix (FastStart Universal SYBR Green Master (Rox), Roche Diagnostics GmbH, Germany) with 25 ng cDNA in triplicates, 25 ng RNA negative controls in duplicates, and water control in duplicate were pipetted onto 96-well plates (ABI MicroAmp® Fast Optical 96-well Reaction Plate with Barcode, 0.1 mL) using a QIAgility pipetting robot (Qiagen AG, Switzerland). The reaction volume was 20 µL. The qPCR reactions (ABI 7500 Fast Real-Time PCR System) started with a holding stage at 50 °C for 2 min, then another holding stage at 95 °C for 10 min. 40 cycles were ran (each 95 °C for 15 sec, followed by 57 °C for 1 min). Melting curves were also analyzed (95 °C for 15 sec, 60 °C for 1 min, then measuring the fluorescence of the amplicons by slowly increasing the temperature with 1 % slope up to 95 °C, finally cooling down to 60 °C for 15 sec). The primers used are listed in the supplementary information (**SI Table 3.2**). The mean normalized expression (MNE) values were calculated by normalizing to the expression levels of elongation factor 1α (*ef1α*). Fold inductions compared to control were obtained by dividing each MNE by the control MNE. One-way ANOVA followed by Dunnett's test was used to evaluate whether the differences are statistically significant (GraphPad Prism 5).

4.2.7 Protein precipitation, digestion and sample desalting

The 35-40 embryos per exposure group were anaesthetized by addition of ice-cold dilution water, counted, collected into 1.5 mL Protein LoBind Tubes (Eppendorf, Vaudaux Eppendorf AG, Switzerland), and snap-frozen in liquid N_2 at 96 hpf. The samples were stored at -80 ºC until further processing. The sample preparation was carried out according to Groh et al.,

2013. Samples were partially thawed on ice, then 400 µL lysis buffer (7 M urea, 2 M thiourea, 1 % CHAPS, 2 % Triton X-100, 100 nM Tris-HCl, pH 8), freshly supplemented with 1x Protease Inhibitor Cocktail (100x, Sigma), was added. The samples were disrupted with 30 strokes using a PTFE pestle. The mixtures were vortexed on ice for 3x10 sec, with 3 min breaks. Next, the samples were sonicated using an ultrasonic processor (LABSONIC® M, Sartorius Stedim Biotech GmbH, Germany; parameters: cycle 1, amplitude 100%, 3x10 sec, with 20-30 sec breaks, all on ice). The lysates were clarified by centrifugation at 13,000 rpm at 4 ºC for 25 min. The supernatant was transferred into a clean LoBind tube. Proteins were precipitated by the addition of MeOH:chloroform:MilliQ water (4:1:3 v:v) into 2x150 µL clarified homogenate. The protein pellets were washed with MeOH. The pellets were air-dried for 4 min, and then wetted by the addition of 6 µL 0.2 M NaOH in order to make the resolubilization easier. The duplicate pellets per sample were dissolved in a total of 50 µL resolubilization buffer (9 M urea, 2 M thiourea, 0.1 M Tris-HCl, pH 8.5) and thus combined. The protein content of the samples was determined by the Bradford method. Afterwards, 2x100 µg protein per sample were treated first with 5 mM tris(2-carboxyethyl)phosphine (TCEP, Sigma) for 30 min at RT in the dark in order to reduce the disulfide bonds. Next, disulfide formation was prevented by alkylation of cysteines using 25 mM iodoacetamide (IAA, Sigma); then the samples were incubated for 30 min at RT in the dark. The digestion mix contained one µg trypsin (sequencing grade, Sigma) and a final concentration of 5 mM $CaCl_2$ dissolved in 0.1 M Tris-HCl (pH 8.5) per 100 µg protein content, and was incubated at 37 ºC overnight (ca 15 h). The reaction was stopped by the addition of 3 µL FA to each sample, then the tryptic digests were centrifuged for 20 min at 13,000 rpm at RT. The digests were combined per sample and desalted using Sep-Pak tC18 (Waters) solid phase extraction (SPE) cartridges with MilliQ water containing 0.1 % FA, and eluted with 80 % ACN / 20% MilliQ water and 0.1 % FA, then evaporated in an Eppendorf Vacuum Concentrator (program V-AL, 30 ºC, duration ca 4-6 h). The cleaned peptides were re-suspended in 200 µL MilliQ water with 0.1 % FA, thus each sample had a total peptide concentration of 1 µg/µL. The samples were filtered (Amicon Ultrafree MC, 0.45 µm pore size) and stored at +4 ºC before the LC-MS/MS analyses.

4.2.8 Developing a targeted proteomics method for simultaneous measurement of several proteins in zebrafish embryos

The targeted proteomics method development workflow was adapted from the Aebersold research group (Lange et al., 2008; Picotti and Aebersold, 2012) and was carried out with minor changes as described in Groh et al., 2013. The protein targets were selected based on literature (**SI Table 4.3**). Up to five proteotypic peptides (PTPs, unique peptides of 8-21 amino acid (AA) length, that have ideal properties for analysis by MS) per protein were

selected with the Sykline software (MacLean et al., 2010). Whenever possible, peptides containing AAs prone to posttranslational modifications were excluded. However, cysteine carbamidomethylation was set as a default due to the cysteine alkylation step before the trypsin digestion. Furthermore, when methionine (M) containing peptides had to be chosen, M oxidation was set as variable, and thus PTPs with and without M oxidation were ordered as well. The uniqueness of each peptide was double-checked by the Ensembl BLAST tool. For each protein, only unique peptides were selected, except for Tnni2a, in which case all the peptides are part of the protein Tnni2b as well. Thus, in this case all PTPs are characteristic for both proteins, and can be called master peptides, measuring the sum of the two proteins at the same time. Later we refer to these results as the abundance of Tnni2ab. Also, the PTP for GR is a master peptide for monitoring both the GRα and GRβ forms. The selected PTPs were commercially synthetized (JPT Technologies, Germany). All of the synthesized peptides were combined into one master mix resulting in approximately 200 fmol/µL/PTP, and a total of 40 nmol/µL peptide concentrations. This master mix was further diluted 100x, 1000x and 10000x for the LC-MS/MS runs.

The LC-MS/MS system consisted of a 1290 Infinity II Binary LC system from Agilent Technologies (Agilent Technologies, Palo Alto, CA, USA) coupled to a 6495 Triple Quadrupole (Agilent) equipped with an ESI source. The chromatographic separation was achieved with a Poroshell EC C18 column (100x2.1 mm, 1.7 µm, Agilent) using a 60 min water/MeOH gradient with 0.2% FA described in Groh et al., 2013. The flow rate was set to 0.15 mL/min, and 10 µL diluted PTP master mix or 20 µL zebrafish embryo digest was injected. The MS source was operated in positive mode. The MS acquisition was conducted in single reaction monitoring (SRM) mode in the method development phase, and dynamic SRM (dSRM) mode for analyzing exposed zebrafish embryo digests. The data acquisition was accomplished with the MassHunter Workstation (Agilent Technologies). The qualitative data analysis was performed with Skyline.

First, the PTP master mix was analyzed for 6 transitions per peptides (all the peptides had doubly charged precursors). In case a peptide contained >15 AAs or had a mass > 750 Da, the triply charged precursor was targeted too, also with 6 transitions. The results were analyzed and the most intense 2-3 transitions per PTP were monitored in control zebrafish embryo digests. Furthermore, PTPs of housekeeping proteins were also monitored in control embryos. The PTPs detected in fish samples and their monitoring parameters used for further analyses of exposed zebrafish embryo digests are listed in **SI Table 4.4**. The two most intense transitions (quantifier and qualifier) of the PTPs were combined into one dSRM method based on the retention times (RTs) using 4 min monitoring windows for each PTP. This dSRM method was used to analyze the abundance of detectable target proteins in embryos exposed to CP or DCF or environmental grab water samples. Protein abundance

was calculated by normalizing the peak area of the target quantitative peak with the housekeeping glyceraldehyde 3-phosphate dehydrogenase (Gapdh) PTP quantitative peak area of the respective sample. Fold inductions compared to the control samples were calculated for each target protein, and these values were analyzed for statistical significance by one-way ANOVA followed by Dunnett's test (GraphPad Prism 5.0).

4.3 Results

4.3.1 Chemical analysis of environmental water samples

GCs, DCF, a progestogen and two pharmaceutical fungicides were detected in the environmental grab water samples (**Table 3.1**). Halometasone was not in the calibration standard mix and thus, its concentration was calculated based on the curve of flumetasone. Flumetasone has exactly the same structure except that it lacks the chlorine in position 2.

Table 4.1: Compounds detected in the grab water samples.

compound	compound group	upstream (ng/L; nM)	effluent (ng/L; nM)	downstream (ng/L; nM)
6α-methylprednisolone	GC	<LOD	2.5; 0.007	1.0; 0.003
halometasone	GC	<LOD	106.1; 0.24*	24.1; 0.05*
diclofenac	NSAID	<LOD	147.5; 0.5	60.9; 0.1
progesterone	progestogen	10.9; 0.03	<LOD	<LOD
clotrimazole	anti-fungal	<LOD	0.3; 0.001	0.2; 0.001
fluconazole	anti-fungal	<LOD	7.8; 0.025	4.0; 0.013

Abbreviations: GC: synthetic glucocorticoid, NSAID: non-steroidal anti-inflammatory drug, LOD: limit of detection
* Concentration calculated based on flumetasone

4.3.2 Expression of inflammatory response-related genes on the mRNA level after exposure to DCF and environmental water samples

In Chapter 3 we have identified six inflammatory-response related genes that were significantly regulated after CP exposure: heat shock protein 70 (*hsp70*), mitogen activated protein kinase 14a (*p38*), nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha b (*IκBα b*), interleukin 8 (*il8*), also called chemokine (C-X-C motif) ligand 8a,

v-fos FBJ murine osteosarcoma viral oncogene homolog Ab (*cfos*) and annexin A1b (*anxa1b*).

The mRNA expression patterns of these six genes after exposure to DCF and environmental samples are shown in **Fig. 4.2**. *Hsp70*, *p38* and *IκBα b* were not regulated after either DCF or environmental water sample exposures (**Fig. 4.2A, B, C**). In contrast, *il8* (**Fig. 4.2D**) was significantly down-regulated after 10 nM DCF exposure. After exposure to the upstream sample, there was no regulation, while after exposure to the downstream sample, a slight up-regulation could be observed. Interestingly, *il8* shows an up-regulation with highly fluctuating results after effluent exposure. In the first biological replicate (R1), it was not regulated compared to the blank control sample, but from the second to the fourth biological replicate (R2 to R4), *il8* showed an increasing tendency for up-regulation. It is noteworthy, that R1 to R4 were exposed in a chronological order, i.e. that R1 was exposed first after collecting and filtering the samples, followed by R2, R3 and R4 in that order. R1 and R2 were exposed during the first week, while R3 and R4 were exposed during the second week after water sample collection. The expression of *cfos* (**Fig. 4.2E**) was clearly up-regulated after 1 and 10 nM DCF exposure. In the upstream exposures, a slight up-regulation was observed that became the strongest for R4. The downstream sample showed an up-regulation as well, but in this case there was no significant difference between R1 to R4. In contrast, the expression pattern of *cfos* after exposure to the effluent sample showed no regulation for R1 and increasing up-regulation from R2 to R4, same as for *il8*. The up-regulation by the effluent sample turned out to be significant. *Anxa1b* (**Fig. 4.2F**) was significantly down-regulated in all replicates after 0.1, 1 and 10 nM DCF exposure. After exposures to the environmental samples, it was down-regulated for R1 and R2 in the upstream, downstream and effluent samples as well. The effect was strongest in the case of the effluent sample. Surprisingly, the results for R3 and R4 were showing the exact opposite. In case of the upstream exposures, there was no regulation compared to the blank control, and in the downstream there was also no regulation for R3, but for R4 *anxa1b* appears to be up-regulated. In the effluent sample, both R3 and R4 were up-regulated by 3.2 and 4.2-fold, respectively.

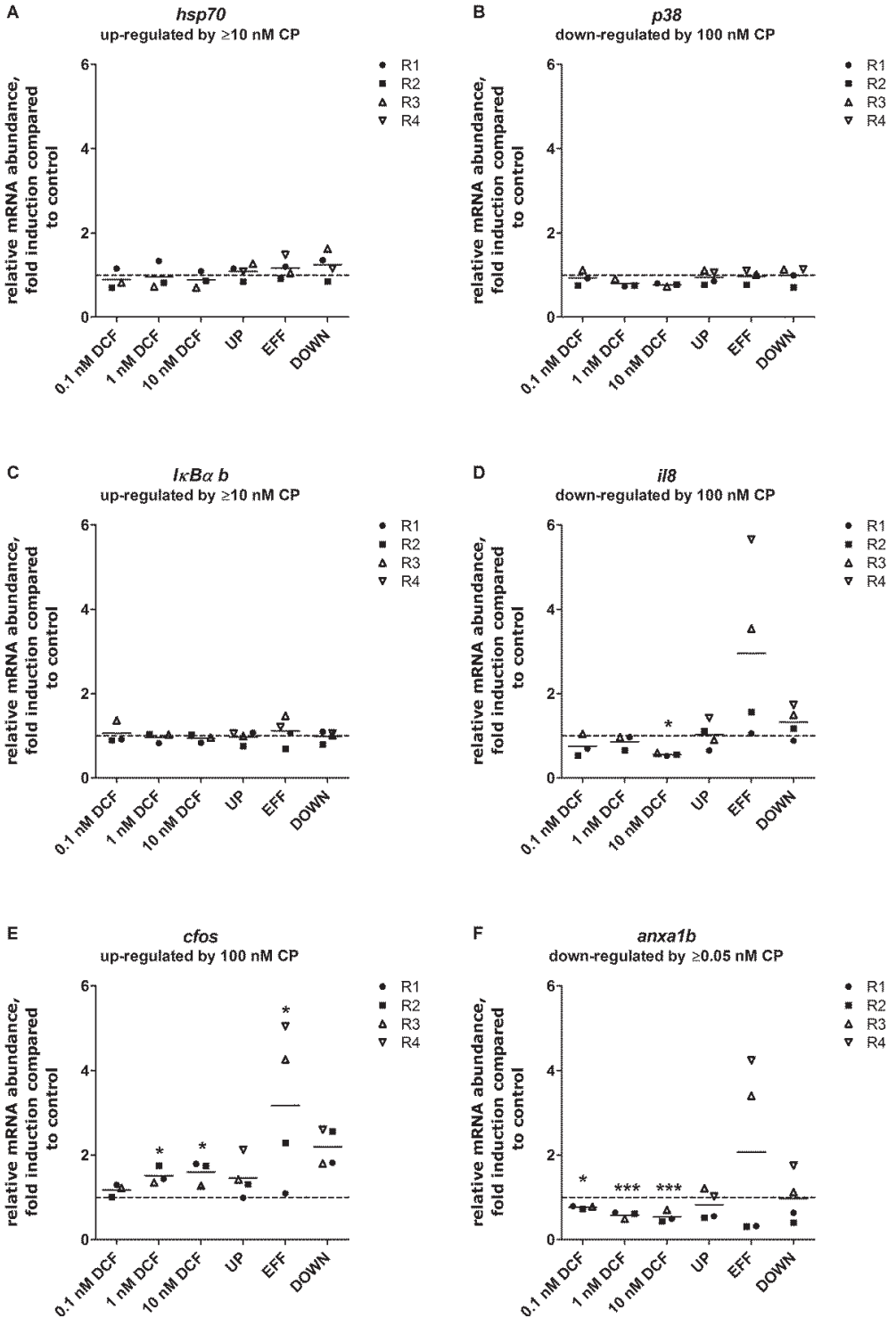


Figure 4.2: mRNA expression patterns of inflammatory response-related genes. *Embryos were exposed to 0.1, 1 and 10 nM diclofenac (DCF) or the filtered environmental grab water samples until 4 days post fertilization. The results of all biological replicates are plotted as fold changes compared to control. The control (fold change=1) is marked by a dashed line. The names of the examined gene transcripts are indicated above the graphs along with a statement regarding the regulation by clobetasol propionate (CP) observed in*

*Chapter 3. N=3 independent experiments for the CP and DCF exposures, and N=4 over the course of two weeks using the same samples for the environmental exposures (UP: upstream, EFF: WWTP effluent, DOWN: downstream). The results were analyzed by one-way ANOVA followed by Dunnett's test, * p < 0.05, ***p < 0.001.*

4.3.3. Development of a targeted proteomics technique for monitoring GC action-related proteins in zebrafish embryos

GC action-related protein targets were selected based on the literature and regulated transcripts. Regulation of these targets has been investigated either after GC exposure or GR knockdown in zebrafish (**SI Table 4.3**). **Fig. 4.3** shows the protein targets grouped by their main physiological roles. Synthetic peptides for each target were analyzed to find the 2-3 most intense transitions. These transitions were then monitored in control zebrafish embryo digests.

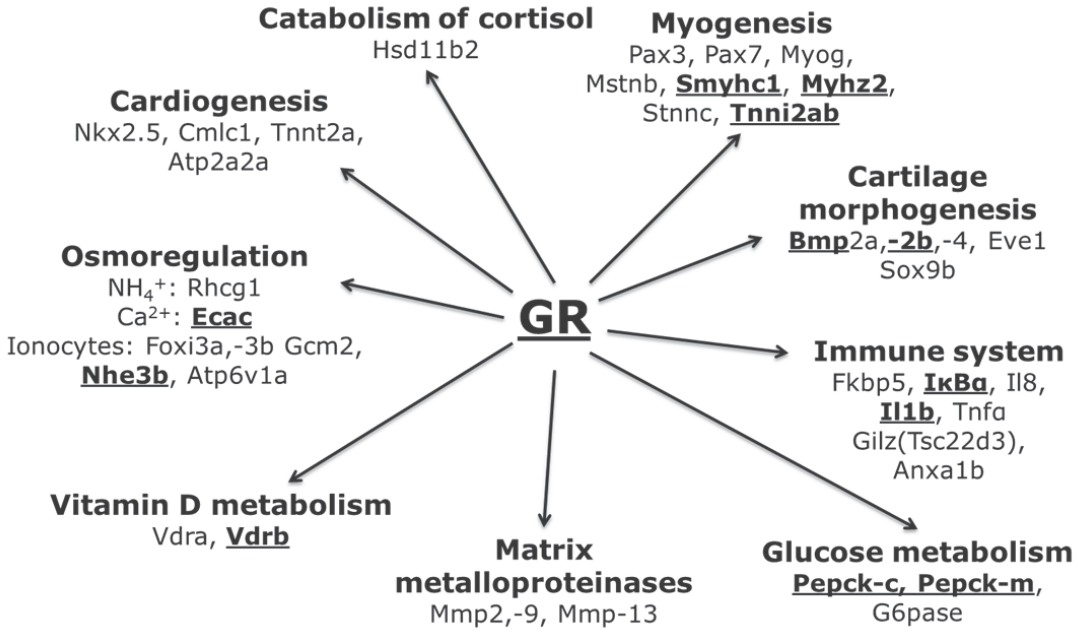


Figure 4.3: Proteins targeted in this study grouped according to their physiological functions. *Underlined are the targets that were detected in zebrafish embryo digests, and analyzed after exposure to CP, DCF and the environmental grab water samples.*

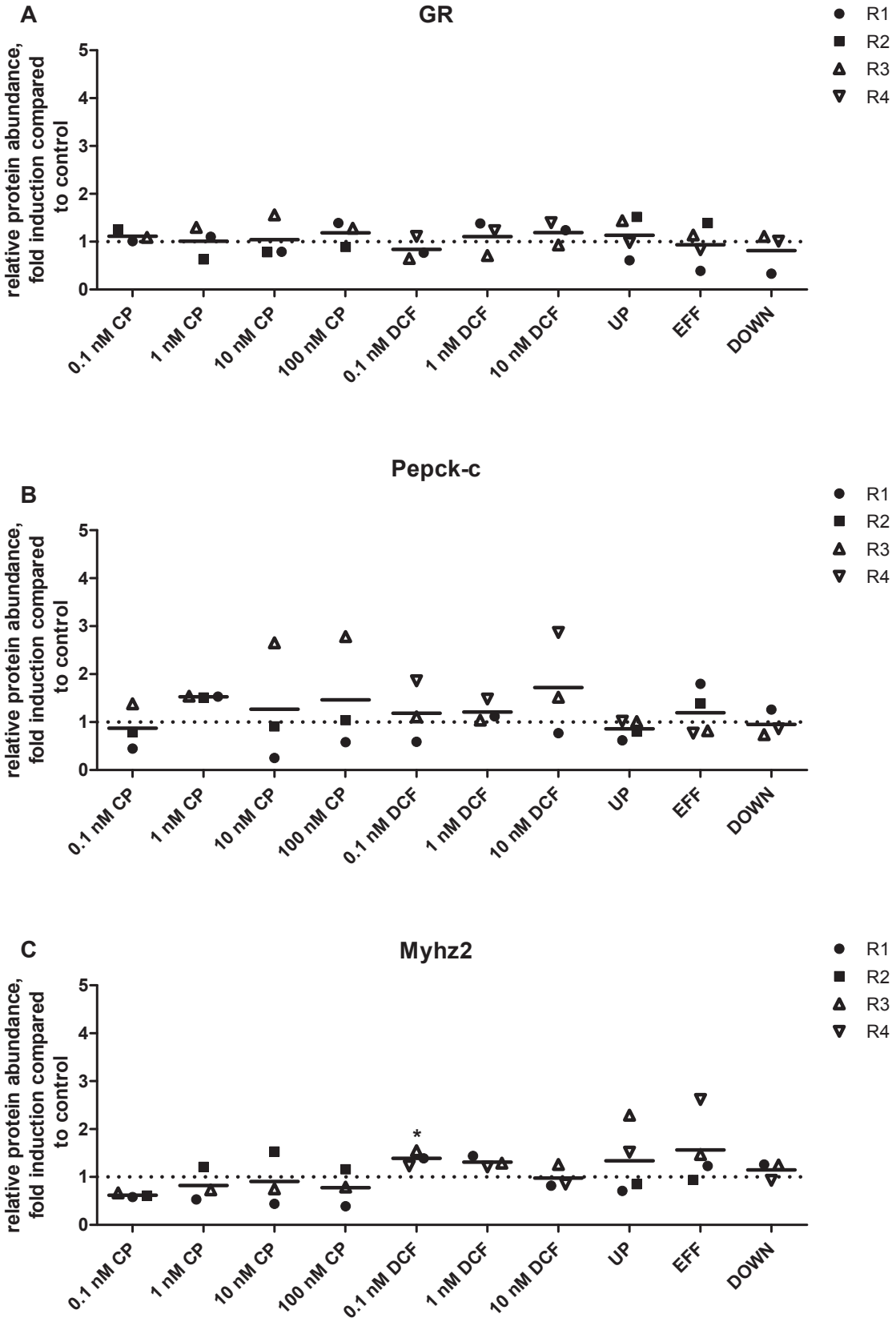
At least one peptide per protein has been detected in the digests for the proteins underlined in **Fig. 4.3**. The transition ratios and the retention times of the detected peptides were in good agreement with those obtained for the synthetic peptides. Three so-called housekeeping proteins were monitored in the digests as well: β-actin (Bact), glyceraldehyde 3-phosphate dehydrogenase (Gapdh) and elongation factor 1α (Ef1α). As these proteins are highly abundant in the samples, the 2-3 most intense transitions were determined experimentally in the digests without using synthetic peptides. Gapdh was used to normalize

the results of the targets after the exposure experiments. The resulting transitions for the 12 GC-action related targets and the three housekeeping peptides were merged into one scheduled SRM method of one hour runtime. The monitored peptides per protein, their transitions and retention times are listed in **SI Table 4.4**. Generally, a bigger variation of the results was observed on the protein level (**Fig. 4.4**) than on the mRNA level.

4.3.4 Expression of GC action-related proteins after exposures to CP, DCF and environmental water samples

Protein abundances varied a lot, and were barely significant, but trends were still visible. Among the protein targets analyzed, the mitochondrial phosphoenolpyruvate carboxykinase (Pepck-m), an important enzyme in gluconeogenesis (**SI Fig. 4.1A**), showed big variance after CP and DCF exposures. The difference in protein expression between the exposures with the grab water samples carried out during the first week (R1 and R2) and during the second week (R3 and R4) is clearly visible. Bone morphogenetic 2b (Bmp2b) (**SI Fig. 4.1B**) was not regulated by CP, while a trend for up-regulation was observed after 10 nM DCF exposure. In the upstream sample, a visible but not statistically significant trend for up-regulation was observed, also for the effluent sample except for R4. The downstream sample was not regulated. Slow myosin heavy chain 1 (Smyhc1) (**SI Fig. 3.1C**) showed a trend of up-regulation but not significantly after 100 nM CP exposure, but did not show any regulation in either the DCF- or environmental water sample-exposed embryos. The master peptide that measured the total abundance of skeletal troponin I type 2a and 2b (Tnni2ab) (**SI Fig. 3.1D**) showed no regulation in any of the treatments. Epithelial calcium channel (Ecac) (**SI Fig. 4.1E**) showed an up-regulation trend after exposure to 1 nM CP, but no regulation was observed in case of other CP concentrations, or after DCF exposure. The trend of up regulation with R1 and R2 and down-regulation with R3 and R4 is also visible here. The expression of vitamin D receptor b (Vdrb) (**SI Fig. 4.1F**) was quite variable, though a trend for up-regulation was visible after exposure to 1 nM CP. After effluent exposure, this protein also showed an opposite expression in R1 and R2 (up-regulation) compared to R3 and R4 (down-regulation). Interleukin-1 beta (Il1b) (**SI Fig. 4.1G**) was slightly up-regulated after 100 nM CP exposure, but was not regulated after DCF or environmental water sample exposures.

GR (**Fig. 4.4A**) showed no regulation even after CP exposure. The cytosolic form of phosphoenolpyruvate carboxykinase (Pepck-c) (**Fig. 4.4B**) showed a trend of up-regulation after 1 nM CP exposure, but otherwise the data was highly variable. Fast muscle myosin heavy chain 2 (Myhz2) (**Fig. 4.4C**) showed a trend of down-regulation after exposure to 0.1 nM CP, a significant up-regulation after exposure to 0.1 nM and a trend of up-regulation after exposure to 1 nM DCF. The higher concentrations of CP and DCF did not cause a differential expression compared to the blank controls. After exposure to the environmental water grab


A
GR
R1
R2
R3
R4
relative protein abundance, fold induction compared to control
0
1
2
3
4
5
0.1 nM CP
1 nM CP
10 nM CP
100 nM CP
0.1 nM DCF
1 nM DCF
10 nM DCF
UP
EFF
DOWN
B
Pepck-c
C
Myhz2
*

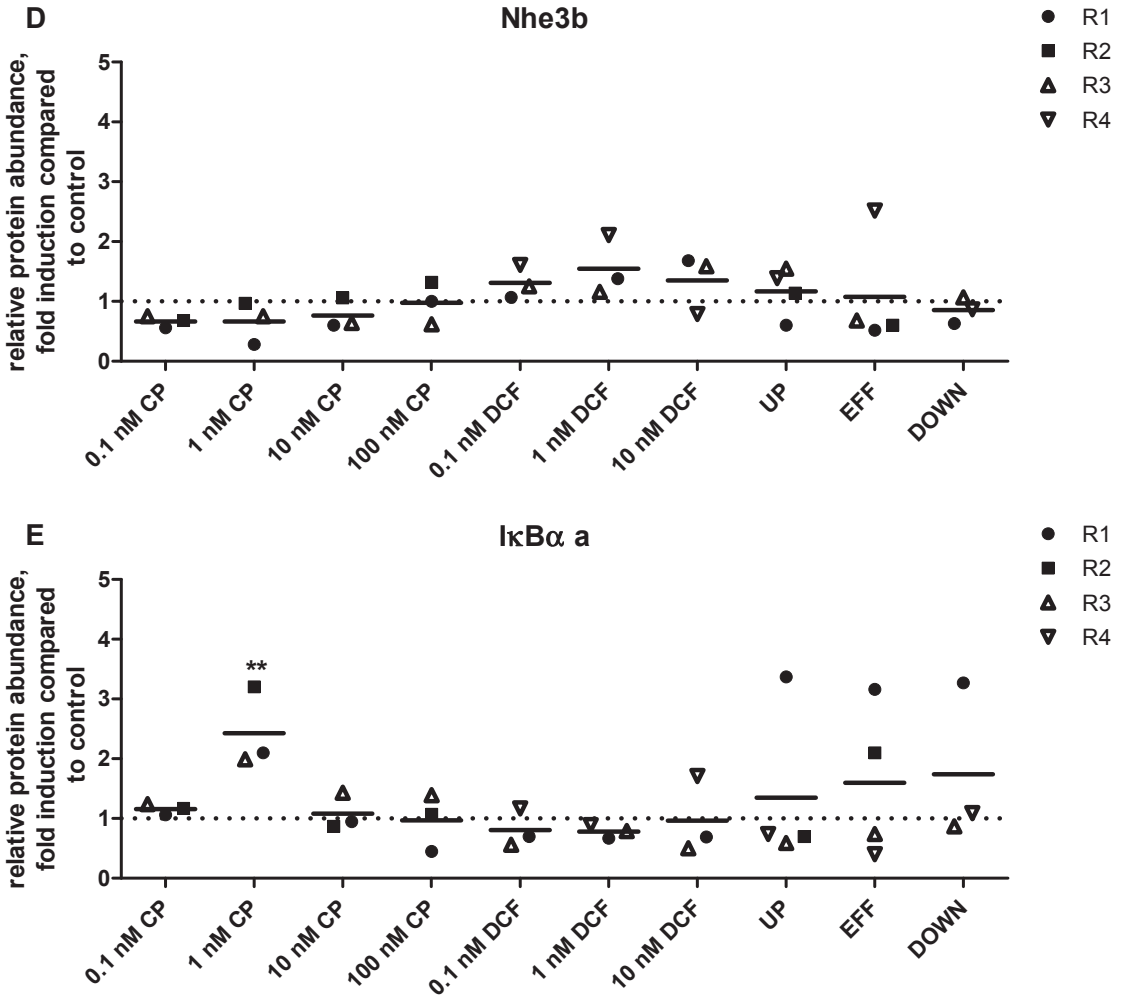


Figure 4.4: Protein expression patterns of selected detectable GC action-related genes. *Embryos were exposed to 0.1, 1, 10 and 100 nM clobetasol propionate (CP); 0.1, 1 and 10 nM diclofenac (DCF) or the filtered environmental grab water until 4 days post fertilization. The results of all biological replicates are plotted as fold changes compared to control. The control (fold change=1) is marked by a dashed line. The names of the examined proteins are indicated above the graphs. N=3 independent experiments for the CP and DCF exposures, and N=4 over the course of two weeks using the same samples for the environmental exposures (UP: upstream, EFF: WWTP effluent, DOWN: downstream). The results were analyzed by one-way ANOVA followed by Dunnett's test, * $p < 0.05$, ** $p < 0.005$.*

samples, up-regulation of Myhz2 was observed in the upstream and effluent samples in the R3 and R4 experiments. This is most obvious for the effluent sample, in which R1 and R2 show a trend of up-regulation, but R3 and R4 a trend of down-regulation. Na^+/H^+ exchanger 3 b (Nhe3b) (**Fig. 4.4D**) was slightly down-regulated after exposure to 0.1 nM CP. The expression was regulated back to control level at 100 nM CP exposure concentration. After DCF treatment, Nhe3b showed a slight up-regulation trend for all the concentrations used in this study. After exposure to the upstream water sample, only R1 showed a slight down-

regulation, R2, R3 and R4 showed no regulation. The downstream sample showed no regulation. However, in the effluent-exposed zebrafish embryos, Nhe3b was down-regulated in the R1, R2 and R3 experiments, but up-regulated in the R4 experiment. The subunit a of IκBα (**Fig. 4.4E**) was significantly up-regulated after 1 nM CP exposure, but was not regulated with the other CP concentrations. In the DCF exposure, IκBα a showed a trend of down-regulation after the 10 nM exposure. Interestingly, this protein was up-regulated in the R1 of all the grab water sample exposures. In case of the effluent exposures, a trend of up-regulation can be seen in R2 too, and a possible down-regulation in R3 and R4.

4.4 Discussion

This study investigated whether biomarker(s) of exposure and effect of GCs or anti-inflammatory drugs can be found in zebrafish embryos. Thus, GC action-related genes, including inflammatory response-related genes, were investigated on the mRNA and protein levels. The expression on the mRNA level after exposure to the model GC CP had been investigated in a previous study (Chapter 3). In order to find specific and sensitive biomarker candidates, the gene expression was examined after exposure to environmentally relevant concentrations of the NSAID DCF. Furthermore, grab water samples taken from a site where GR-active compounds and DCF had been detected before (Tousova et al., in preparation; Chapter 2) were used for embryo exposures and validation of the observed effects in a complex matrix.

4.4.1 Chemical analysis

The grab water sample collected from upstream of a WWTP contained the steroid hormone progesterone that interferes with the regulation of genes through the HPI axis in zebrafish embryos (Liang et al., 2015). The effluent of the WWTP and the downstream site contained the same compounds (**Table 4.1**). The pharmaceuticals detected at the downstream site had an approximately 2-5 times lower concentration compared to the effluent, which likely is due to the dilution in the river. GCs are characterized by their relative potency (REP) to activate the GR, and the REP value of the GC dexamethasone (DEX) was chosen as 1. Of the two GCs detected in the effluent and downstream samples, 6α-methylprednisolone has a relative potency compared to dexamethasone of 0.54 (Macikova et al., 2014) making it 70 times less potent than CP. The other GC, halometasone, has, unfortunately, not yet been investigated for its REP. DCF was also detected with a downstream concentration equal to the lowest exposure concentration used in this study (0.1 nM), and an effluent concentration of 0.5 nM. Thus, the detected GCs and DCF in the effluent and downstream samples are comparable to

those used in the single compound exposures, making these environmental samples ideal for checking whether the effects are still observable in a complex matrix.

4.4.2 mRNA analysis

The expression of inflammation-related genes, already shown to be regulated after CP exposure on the mRNA level (Chapter 3), showed similarities but also some differences in expression pattern after DCF exposure.

We found that DCF did not regulate *hsp70* (**Fig. 4.2A**) a gene connected to cellular defense mechanisms, which is also part of the cytosolic GR complex (Hutchison et al., 1994). Compared to the response of embryos after CP exposure, it can be concluded that *hsp70* is regulated by CP but not by DCF. The regulation by CP was only seen in higher than environmentally relevant concentrations, and indeed we found no regulation after the effluent and downstream exposure (**Fig. 4.2A**). In another study, regulation was observed after exposure up to 6.3 µM DCF in zebrafish embryos compared to blank controls (Hallare et al., 2004), which was in accordance with our results. It seems that no other compound potentially present in the samples regulated the expression of this gene, although it has been proposed that *hsp70* is a biomarker for the exposure to several environmental pollutants in fish (Jing et al., 2013; Maradonna and Carnevali, 2007; Xing et al., 2015).

The trend for down-regulation of *p38* after DCF exposure (**Fig. 4.2B**), which was similar to the regulation after CP exposure but not significant, may be an indication for the inhibition of the MAPK pro-inflammatory pathway. Thus, it seems that the MAPK pathway is inhibited by both steroidal and non-steroidal anti-inflammatory drugs, but that there is no response at environmentally relevant concentrations, as seen after the water sample exposures.

Our results indicate that I*κBα*, which inhibits the NFκB pro-inflammatory pathway (Jacobs and Harrison, 1998; Verma et al., 1995), not regulated by an NSAID (**Fig. 4.2C**). However, it was induced by CP at higher than the environmentally relevant concentrations in zebrafish embryos. Previously, Takada et al. found strongly increased abundance of IκBα protein in a human leukemic cell line KBM-5 after induction by tumor necrosis factor (TNF) and exposure to ≥10 µM DEX and ≥500 µM DCF (Takada et al., 2004). Our study also supports the finding that IκBα is more susceptible to regulation by CP than DCF based on effective exposure concentration. However, based on the lack of regulation after grab water sample exposures, this gene is also not a suitable candidate biomarker.

Our results showed a similar regulation of *il8*, an inflammatory chemokine after both DCF (**Fig. 4.2D**) and CP (Chapter 3) exposure. Thus, GCs and the COX-inhibitor DCF both down-regulate this gene in teleost fish. *Il8* was shown to be down-regulated in the synovial fluid of arthritis-induced rabbits treated with 5mg/kg/8h DCF (Lopez-Armada et al., 2002). Therefore,

DCF exposure down-regulates *il8* both in fish and mammals. Our results also indicate an induced expression of this gene in the effluent- and downstream-exposed zebrafish embryos. The intriguing response pattern of *il8* due to the time of analysis after sample collection (one week versus two weeks) indicates that some chemical degradation could have occurred, which resulted in product(s) that affected the expression of this gene. Microbiological cause is unlikely to play a role, as the water samples were filtered two times through a 0.2 µm pore-sized filter in an attempt to eliminate bacteria. The induction of *il8* may be due to the degradation of other environmental pollutants present in the samples. Up-regulation of *il8* have been demonstrated in fish after exposure to environmental pollutants such as copper (Jiang et al., 2016), or atrazine and chlorpyrifos (Chen et al., 2014), but also due to hypoxic stress (Basu et al., 2016).

We observed the induction of *cfos* after both DCF (**Fig. 3.2E**) and CP exposure (Chapter 3). Based on our results, *cfos* is regulated after 100-times lower exposure concentration of DCF compared to CP. This gene is a part of activator protein 1 (AP-1) (Guha and Mackman, 2001), a downstream transcription factor in the MAPK pathway, but it is also used as a biomarker for neuronal activity (Dragunow and Faull, 1989). The expression of *cfos* in the spinal cord has been shown to be induced by dexamethasone and DCF in inflammation-induced rats and the effect was enhanced after co-administering the two drugs (Buritova et al., 1996). However, we cannot be sure if this up-regulation pattern means the induction of AP-1 and/or a higher neuronal activity in the zebrafish embryos. The trend of induction in the downstream sample and the significant increase in the effluent-exposed embryos may be due to the presence of both GCs and DCF.

The significant *anxa1b* down-regulation after ≥0.1 nM DCF exposure (**Fig. 4.2F**), and the similar finding after ≥0.05 nM CP (Chapter 3) exposure are intriguing because they show an opposite way of regulation compared to humans, since *anxa1* transcripts were found to be up-regulated in the peripheral blood mononuclear cells of overweight patients after administration of 150 mg/day DCF for 9 days in a study investigating inflammatory modulation (van Erk et al., 2010). The function of *anxa1* in mammals is the inhibition of phospholipase A2, and thus the whole arachidonic acid pathway as depicted in **Fig. 4.1.** As already mentioned, DCF inhibits the COX enzymes that are within this pathway. As discussed in chapter 3, zebrafish has three *anxa1* transcript variations: *anxa1a*, *-b* and *-c* (Farber et al., 2003). Thus, *anxa1b* may have a function in fish that differs from that in mammals, and thus be regulated in a different manner. Because the COX enzymes are downstream from *anxa1,* the regulation of this gene by DCF may be a result of systemic negative feedback. The observed regulation in the upstream sample might be due to the detected progesterone. However, the effect of progesterone on the regulation of *anxa1* has not yet been investigated. The down-regulation in the effluent sample was stronger than in

the downstream sample after the R1 and R2 experiments, consistent with the GC and DCF concentrations detected in them. In the effluent sample, an increasingly strong up-regulation pattern was observed in R3 and R4, a completely opposite effect compared to R1 and R2. This supports our hypothesis that some kind of chemical degradation may have taken place during the two weeks when the samples were used for zebrafish embryo exposures. This chemical degradation possibly resulted in the formation of products that affected the expression of *il8*, *cfos* and *anxa1b* genes on the mRNA level.

4.4.3 Protein analysis

To extend knowledge on the regulation of GC action-related genes from the mRNA to the protein level, 40 protein targets were identified, most of them having been examined previously after either GC exposure or GR knockdown in zebrafish (**SI Table 4.3**). The protein target list included Anxa1b, Il8 and IκBα proteins, based on their significant regulation of mRNA abundance after CP exposure as shown in Chapter 3. Many of the targets are transcription factors, or other low abundant proteins, requiring the use of targeted proteomics. Zebrafish embryo digests are moreover highly complex samples, reducing the chance of finding low abundant proteins through cellular fractionation but increasing the risk of ion suppression. These conditions may explain why only 12 of the 40 target proteins were detected in the zebrafish embryo digests using SRM.

Our results showed no regulation of the GR protein (**Fig 4.4A**), However, GR transcripts were observed to be up-regulated after exposure to 0.2-19.2 nM beclomethasone dipropionate (BDP) for 21 days in the liver tissue of adult fathead minnows (*Pimephales promelas)* (Kugathas et al., 2013; Margiotta-Casaluci et al., 2016) and down-regulated after 55.2 µM cortisol exposure from 0 to 1 dpf in zebrafish embryos (Cruz et al., 2013; Lin et al., 2011). Since different GCs have been used in these studies including ours, a comparison of the results requires taking into account the GR-activity of the GCs used based on their relative potencies (REP). The REP value of BDP is 0.53. However, its active metabolite, beclomethasone-17-monopropionate, has a higher REP value of 13 (Margiotta-Casaluci et al., 2016), while cortisol has a REP value of 0.036 (Macikova et al., 2014). Thus, the GR induction in fish mentioned above was seen after exposure to 2.6-250 nM DEX-equivalents (DEX-EQs), and suppression after 1990 nM DEX-EQs. The REP value of CP is 38 (Macikova et al., 2014), thus our exposure experiments were equal to 3.8-3800 nM DEX-EQs, covering the whole range. The zebrafish GR is present in the form of two splice variants, the cytosolic GR α and the GR β that mostly resides in the nucleus and cannot be activated by ligands, similarly to humans (Schaaf et al., 2008; Schoonheim et al., 2010). Despite the high similarity of the α and β variants, we managed to find PTPs for both proteins, however they were not detected in control embryo samples, only a master peptide

monitoring both. Based on the studies mentioned above, we should have seen an induction of GR in the lower concentrations, and down-regulation in the higher ones due to systemic negative feedback. So a possible explanation for not detecting any regulation could be that we couldn't detect the regulation of the two splice variants independently with the master peptide.

Pepck protein levels were not regulated after either CP or DCF exposures (**Fig. 4.4B and SI Fig. 4.1A**) in our study. Nevertheless, the different expression pattern correlating with the age of the effluent sample after collection was observed with Pepck-m (**SI Fig. 4.1A**). However, Pepck-c and Pepck-m are key enzymes in mammal gluconeogenesis and have been shown to be regulated by GCs (Hanson and Reshef, 1997). Pepck expression on the mRNA level was induced after 0.1 nM BDP exposure in rainbow trout (*Oncorhynchus mykiss)* liver tissue (Carney Almroth et al., 2014), and after 0.2 nM BDP exposure in fathead minnow liver tissue (Kugathas et al., 2013; Margiotta-Casaluci et al., 2016). The transcripts of *pepck-m* showed significant up-regulation after exposure to ≥0.05 nM DEX, prednisolone and triamcinolone from 3 to 123 hpf in zebrafish embryos (Chen et al., 2016). The expression of these genes has not been examined after DCF exposure, although in one study they did see an inhibitory effect on gluconeogenesis in the livers of fasted rats treated with 0.01-0.1 mM DCF (Petrescu and Tarba, 1997). Even though in other studies induction of gluconeogenesis was observed, our results do not support these findings.

It has been shown that GR signaling is essential for muscle development in zebrafish embryos (Nesan et al., 2012). Thus, we examined the expression of one bone morphogenetic protein, Bmp2b and three muscle proteins, Smyhc1, Tnni2ab and Myhz2. Our results indicated no significant changes of the levels of the proteins Bmp2b, Smyhc1 and Tnni2ab after either CP or DCF exposure (**SI Fig. 4.1B, C, D**). Bmp2b showed the different expression patterns mentioned earlier (R1-R3 versus R4), caused by possible chemical degradation of the effluent sample. The lack of regulation of Tnni2ab may be due to the fact that we monitored the total abundance of Tnni2a and Tnni2b proteins with our master peptide (**SI Fig. 4.1D**). These have previously been investigated on the mRNA level, after GR morpholino knockdown in zebrafish embryos at 24 and 36 hpf (Nesan et al., 2012). Among them, *bmp2b* transcripts were decreased, *smyhc1* and *tnni2a* were up-regulated, and *myhz2* did not show significant changes after GR knockdown. Thus, when GR is present and down-regulated, similar trends should be observed, while after up-regulation of the GR the opposite effects should be seen. Therefore, the importance of GR signaling in the muscle development of zebrafish embryos is not supported by our results, however this does not mean that GR signaling is not important. In their study Nesan et al also found that *myhz2* did not show any difference after GR knockdown in zebrafish embryos. Myhz2 protein seemed to be slightly down-regulated after exposure to 0.1 nM CP, and it was significantly up-regulated

after exposure to 0.1 nM DCF (**Fig. 4.4C**). Based on our chemical data, the downstream sample contained exactly the same concentration of DCF, but we did not see any regulation of this protein. Thus, this regulatory effect by the NSAID but not with the steroidal anti-inflammatory drug was possibly masked by the complex matrix of the environmental water sample.

The expression pattern of Ecac, a protein playing a role in Ca^{2+} uptake and Vdrb, one of the vitamin D_3 receptors were similar in our study (**SI Fig. 4.1E, SI Fig. 4.1F**) but not significant, even though both have been shown to be regulated by cortisol in zebrafish embryos (Cruz et al., 2013; Lin et al., 2011).

The same group showed Nhe3b, a gene marker for H^+-ATPase rich cells (HRCs), was down-regulated after exposure to cortisol on the mRNA level in zebrafish embryos (Cruz et al., 2013). A similar suppression pattern was observed after CP and effluent sample exposure in our study, while DCF slightly up-regulated this protein (**Fig. 4.4D**). This protein needs further investigation as candidate biomarker of GC exposure and effects, because our experiments showed distinct trends but no significant results after three biological replicates.

We found no significant regulation of Il1b, an inflammatory cytokine, after CP, DCF or environmental water sample exposures (**SI Fig. 4.1G**) even though the *il1b* transcript was found to be down-regulated after GC exposure in mammals (Waterman et al., 2006) and in zebrafish embryos after 100 µM DEX exposure (Schaaf et al., 2009). It was also found to be down-regulated after DCF exposure in human cells (Gonzalez et al., 1994; Henrotin et al., 2001; Kusuhara et al., 1997). This suggests that Il1b does not respond in zebrafish embryos at the low concentrations found in the environment.

The mRNA and protein level expression of IκBα (**Fig. 4.2C, 4.4E**) showed that CP regulates IκBα after exposure to nanomolar concentrations, but not DCF. Furthermore, *IκBα* on the mRNA level showed significant up-regulation by 10 and 100 nM CP, while the protein level was significantly increased by 1 nM, but not by 10 and 100 nM CP exposures. No clear correlation between mRNA and proteins could be found. Nonetheless, the effect is not robust enough in complex environmental samples, and therefore, IκBα is not suitable as a biomarker for environmental GC exposure.

4.5 Conclusions

In summary, we showed that some of the selected genes were regulated on the transcript level by CP only (*hsp70*, *p38* and *IκBα*) but only at concentrations above what is found in the environment. Some of the transcripts were regulated by both steroidal and non-steroidal anti-inflammatory action (*il8*, *cfos* and *anxa1b*) including environmental samples containing both CP and DCF.

On the protein level we could show that Myhz2 was regulated only after DCF exposure but not after exposure with the environmental samples. IκBα is the one target gene that was regulated on both the mRNA and protein level, but here too no response was observed after exposure to the grab water samples.

Out of the selected genes, *cfos* and *anxa1b* showed similar response in the environmental samples as after CP and DCF exposure. However, the effect behind *cfos* regulation is not clear and needs further investigation. While *anxa1b* is clearly linked to the inflammatory response, its inversed regulation compared to mammals needs to be analyzed further. Nevertheless, as it responded sensitively to all exposure scenarios it remains a candidate biomarker for detecting anti-inflammatory effects.

Contributions: I carried out all the zebrafish embryo exposures, and qPCR analyses. I also conducted the environmental grab water collection, sample preparation and targeted chemical analysis, furthermore the targeted proteomics method development and measurements, all of this with the great assistance of René Schönenberger.

4.6 Supplementary information

SI Table 4.1: *Parameters of the grab water samples collected on 30.03.2016*

	Upstream	Effluent	Downstream
Collection time (hh:mm)	17:10-17:15	17:45-17:50	17:30-17:35
Temperature (^{0}C)	12.6	12.4	12.5
O_2 content (mg/L)	12.78	3.95	10.84
Conductivity (µS/cm)	591	1057	706
pH	8.45	7.22	8.04

SI Table 4.2: Overview of primers used in this study for qPCR. *F and R stand for forward and reverse directions.*

Gene	Name	Direction	Sequence (5' → 3')	Acession number	Reference
annexin A1b, lipocortin 1	*anxa1b*	F	AAACTACCCTGCTTGCCCTTT	NM_181759.1	Chapter 3
		R	GATGCTCTCCTGCCTCGTAT		
chemokine (C-X-C motif) ligand 8a	*il8*	F	AAGCCGACGCATTGGAAAAC	XM_001342570.5	Mottaz et al., under review
		R	GTTGTCATCAAGGTGGCAATGA		
nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha b	*IκBα b*	F	TTTGGACAGCGGGTTGGATT	NM_199629.1	Chapter 3
		R	AGTGCAGTCTGTCTCTGGTTG		
v-fos FBJ murine osteosarcoma viral oncogene homolog Ab	*cfos*	F	CCGCACACAAACCCATCTG	NM_205569.1	Jin et al., 2010
		R	GGTCGTGATTGATGTGTTTGGT		
heat shock cognate 70-kd protein, tandem duplicate 1	*hsp70*	F	CAACAACCTGCTGGGCAAA	NM_131397.3	Keegan et al., 2002
		R	GCGTCGATGTCGAAGGTCA		
mitogen-activated protein kinase 14a	*p38*	F	AACGTGACGGTGGACATTTG	NM_131722.1	Sheng et al., 2016
		R	TGGGCATCTGAGGAAGTGAG		
eukaryotic translation elongation factor 1 alpha 1	*ef1α*	F	GGAGTGATCTCTCAATCTTGAAACT	NM_131263.1	Mottaz et al., under review
		R	TGTGGGTCTTTTCCTTTCCCA		

SI Table 4.3: Overview of the selected zebrafish protein targets. *The exposure or GR knockdown experiments were carried out in zebrafish embryos.*

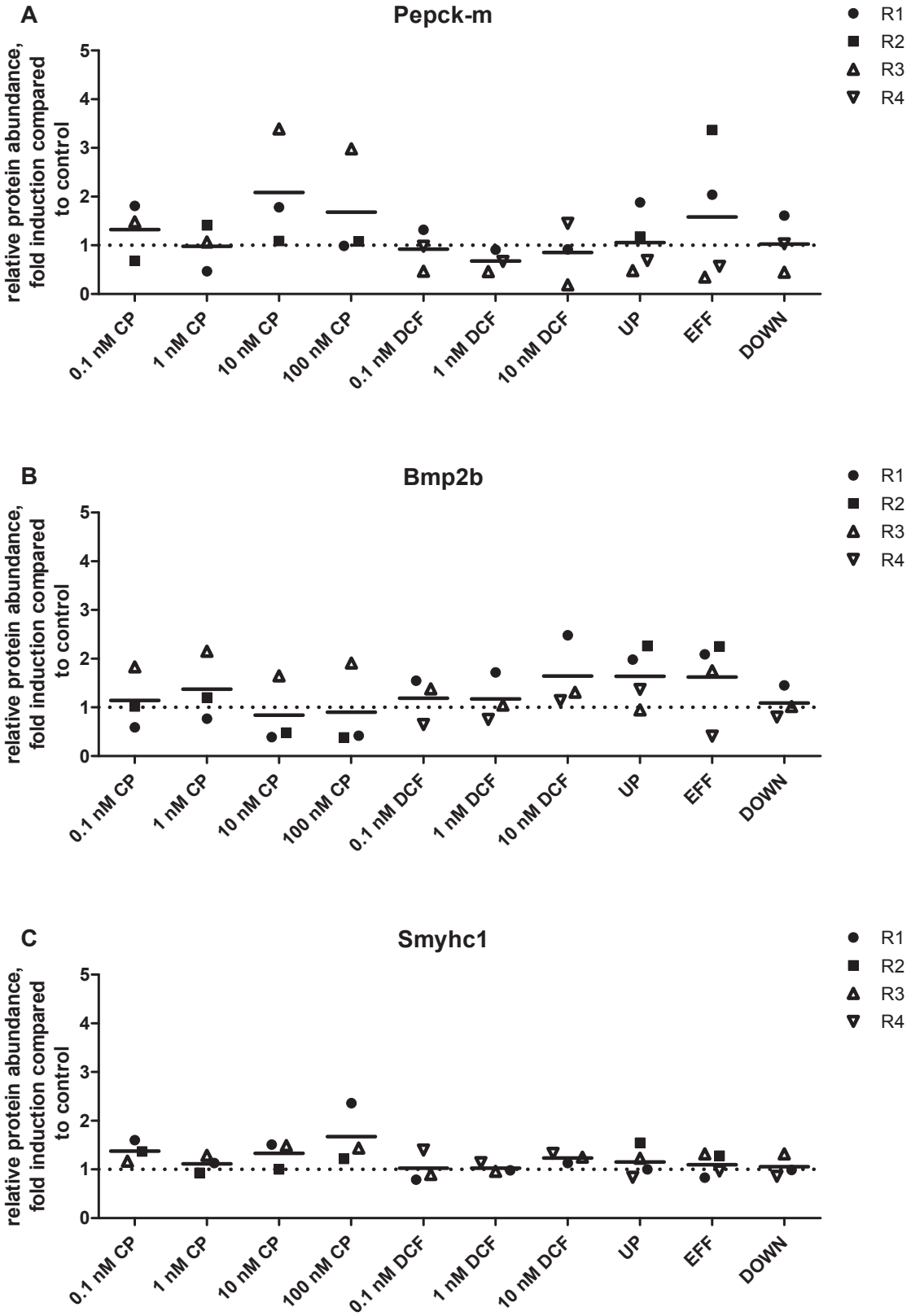
Protein	Name	Accession number	Experiment	Regulation on mRNA level	Reference
paired box protein Pax-3, tr f	Pax3	NP_571352	GR knockdown	down	Nesan et al., 2012
myogenin, tr f	Myog	NP_571081	GR knockdown	up	Nesan et al., 2012
myostatin b, tr f	Mstnb	NP_571094	GR knockdown	down	Nesan et al., 2012
slow myosin heavy chain 1	Smyhc1	NP_001018343	GR knockdown	up	Nesan et al., 2012
fast muscle myosin heavy chain 2	Myhz2	NP_694514	GR knockdown	no regulation	Nesan et al., 2012
slow muscle troponin C	Stnnc	NP_001002085	GR knockdown	up	Nesan et al., 2012
skeletal troponin 1 type 2a	Tnni2a	NP_001009901	GR knockdown	up	Nesan et al., 2012
bone morphogenetic protein 2a	Bmp2a	NP_571434	GR knockdown	down	Nesan et al., 2012
bone morphogenetic protein 2b	Bmp2b	NP_571435	GR knockdown	down	Nesan et al., 2012
bone morphogenetic protein 4	Bmp4	NP_571417	GR knockdown	down	Nesan et al., 2012
even-skipped-like1	Eve1	NP_571189	GR knockdown	down	Nesan et al., 2012
SRY-box-containig gene 9b, tr f	Sox9b	NP_571719	exposure: 1 µM beclomethasone dipropionate	up (non-significant)	Mathew et al., 2007
FK506 binding protein 5	Fkbp5	NP_998314	exposure: 1 µM beclomethasone dipropionate	up	Mathew et al., 2007
nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	IκBα	NP_998349 (a subunit) NP_955923 (b subunit)	exposure: 100 µM dexamethasone ≥10 nM CP	up	Schaaf et al., 2009 Chapter 3
chemokine (C-X-C motif) ligand 8b, duplicate 1 precursor	Il8	NP_001314914	exposure:100 µM dexamethasone 100 nM CP	down	Schaaf et al., 2009 Chapter 3
interleukin-1 beta	Il1b	NP_998009	exposure: 100 µM dexamethasone	down	Schaaf et al., 2009
tumor necrosis factor, alpha	Tnfα	NP_998024	exposure: 100 µM dexamethasone	down	Schaaf et al., 2009
TSC22 domain family protein 3	Gilz	NP_956863	exposure: 1 µM beclomethasone	up	Mathew et al., 2007

			dipropionate		
annexin A1b	Anxa1b	NP_861424	exposure: 0.05 nM CP 250nM beclomethasone (heart) 1 µM GCs*	down up	Chapter 3 Huang et al., 2013 Sengupta et al., 2012
phosphoenolpyruvate carboxykinase, cytosolic [GTP]	Pepck-c	NP_999916	exposure: 100 µM dexamethasone	up	Schaaf et al., 2009
phosphoenolpyruvate carboxykinase [GTP], mitochondria	Pepck-m	NP_998357	exposure: 100 µM dexamethasone 0.05-50 nM dexamethasone, prednisolone, triamcinolone	up	Schaaf et al., 2009 Chen et al., 2016
glucose-6-phosphatase a, catalytic subunit, tandem duplicate 1 isoform 1	G6Pase	NP_001003512	-		
matrix metalloproteinase 2	Mmp2	NP_932333	exposure: 255/276 µM dexamethasone/cortisol	up	Hillegass et al., 2008
matrix metalloproteinase 9	Mmp9	NP_998288	exposure: 255/276 µM dexamethasone/cortisol	up	Hillegass et al., 2008
matrix metalloproteinase 13	Mmp13	NP_958911	exposure: 255/276 µM dexamethasone/cortisol	up	Hillegass et al., 2007
vitamin D receptor A	Vdra	NP_570994	exposure: 55.2 µM cortisol GR knockdown	up down	Lin et al., 2011
vitamin D receptor B	Vdrb	NP_001153457	exposure: 55.2 µM cortisol GR knockdown		Lin et al., 2011
ammonium transporter Rh type C-like 2	Rhcg1	NP_996965 (record removed)	exposure: cortisol 500 nM	-	Kumai et al., 2012
transient receptor potential cation channel subfamily V member 6 (epithelial calcium channel)	Ecac	NP_001001849	exposure: 55.2 µM cortisol	up	Cruz et al., 2013; Lin et al., 2011
forkhead box I3a	Foxi3a	NP_944599	exposure: 55.2 µM	up	Cruz et al., 2013

			cortisol		
forkhead box protein I1	Foxi3b	NP_944600	exposure: 55.2 µM cortisol	up	Cruz et al., 2013
chorion-specific transcription factor GCMb (glial cells missing 2)	Gcm2	NP_001005603	exposure: 55.2 µM cortisol	up	Cruz et al., 2013
solute carrier family 9, subfamily A (NHE3, cation proton antiporter 3), member 3, tandem duplicate 2 precursor	Nhe3b	NP_001106951	exposure: 55.2 µM cortisol	up	Cruz et al., 2013; Lin et al., 2015
V-type proton ATPase catalytic subunit A	Atp6v1a	NP_957429	exposure: 55.2 µM cortisol	up	Cruz et al., 2013; Lin et al., 2015
homeobox protein Nkx-2.5	Nkx2.5	NP_571496	microinjection: cortisol 32 pg/embryo	down	Nesan and Vijayan, 2012
cardiac myosin light chain-1	Cmlc1	NP_571767	microinjection: cortisol 32 pg/embryo	down	Nesan and Vijayan, 2012
troponin T, cardiac muscle	Tntt2a	NP_690853	microinjection: cortisol 32 pg/embryo	down	Nesan and Vijayan, 2012
sarcoplasmic/endoplasmic reticulum calcium ATPase 2	Atp2a2a	NP_957259	microinjection: cortisol 32 pg/embryo	down	Nesan and Vijayan, 2012
glucocorticoid receptor (α and β)	GR	NP_001018547 (α); ABR88076 (β)	exposure: 55.2 µM cortisol	down	Cruz et al., 2013; Lin et al., 2011
corticosteroid 11-beta-dehydrogenase isozyme 2	Hsd11b2	NP_997885	exposure: 55.2 µM cortisol	down	Lin et al., 2011
glycaraldehyde-3-phosphate dehydrogenase	Gapdh	NP_001108586			
actin, cytoplasmic 1 (β-actin)	Bact	NP_571106			
elongation factor 1 alpha	Ef1α	NP_571338			

SI Table 4.4: Parameters of the monitored peptides in the LC-MS/MS runs.

Name	Peptide sequence	Monitored transitions (m/z)	Retention time (min) Standard (extract)
Smyhc1	K.TNSEVAQWR.T [1366, 1374]	$545.74^{2+} \rightarrow 560.29^{+}$ $545.74^{2+} \rightarrow 659.36^{+}$	9.7
Myhz2	K.VTVVTLDTQTEK.V [59, 70]	$667.36^{2+} \rightarrow 935.47^{+}$ $667.36^{2+} \rightarrow 1034.54^{+}$	14.7
Tnni2ab	R.MSADAMLQALLGSK.H [112, 125]	$718.37^{2+} \rightarrow 960.55^{+}$ $718.37^{2+} \rightarrow 829.51^{+}$	38.8
Bmp2b	R.TDTNFLNEFELR.L [43, 54]	$749.86^{2+} \rightarrow 920.48^{+}$ $749.86^{2+} \rightarrow 807.40^{+}$	34.9 (35.2)
IκBα	QTALHLAVVTEQPQ**M**VER	$689.36^{2+} \rightarrow 775.38^{+}$ $689.36^{2+} \rightarrow 903.44^{+}$	19.4 (20.6)
Il1b	R.IINFELQDK.V [260, 268]	$560.31^{2+} \rightarrow 893.44^{+}$ $560.31^{2+} \rightarrow 632.33^{+}$	20.8 (22.6)
Pepck-c	K.TVIVTAEQR.D [91, 99]	$508.79^{2+} \rightarrow 604.30^{+}$ $508.79^{2+} \rightarrow 816.46^{+}$	8.7
Pepck-m	K.TGSFLWPGFGENAR.V [532, 545]	$760.87^{2+} \rightarrow 847.41^{+}$ $760.87^{2+} \rightarrow 1033.49^{+}$	31.5 (33.0)
Vdrb	K.VSEVLQAYIR.A [355, 364]	$589.33^{2+} \rightarrow 650.36^{+}$ $589.33^{2+} \rightarrow 763.45^{+}$	23.6 (24.2)
Ecac	R.VNDIPLFSATK.E [40, 50]	$602.83^{2+} \rightarrow 763.43^{+}$ $602.83^{2+} \rightarrow 991.55^{+}$	24.0 (24.6)
Nhe3b	K.INAAEIIK.G [258, 265]	$436.27^{2+} \rightarrow 644.40^{+}$ $436.27^{2+} \rightarrow 758.44^{+}$	17.2 (16.4)
GR	VLLLLNTVPK	$555.37^{2+} \rightarrow 897.58^{+}$ $555.37^{2+} \rightarrow 671.41^{+}$	30.3 (29.3)
Gapdh	R.GASQNIIPASTGAAK.A [198, 212]	$693.37^{2+} \rightarrow 702.38^{+}$ $693.37^{2+} \rightarrow 815.46^{+}$	(11.4)
Bact	K.SYELPDGQVITIGNER.F [238, 253]	$597.64^{3+} \rightarrow 689.36^{+}$ $597.64^{3+} \rightarrow 802.44^{+}$ $895.95^{2+} \rightarrow 1298.67^{+}$ $895.95^{2+} \rightarrow 901.51^{+}$	(27.5)
Ef1α	R.EHALLAFTLGVK.Q [134, 145]	$649.88^{2+} \rightarrow 664.40^{+}$ $649.88^{2+} \rightarrow 848.52^{+}$	(30.4)


A
Pepck-m
relative protein abundance, fold induction compared to control
0
1
2
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0.1 nM CP
1 nM CP
10 nM CP
100 nM CP
0.1 nM DCF
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UP
EFF
DOWN
R1
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R3
R4
B
Bmp2b
relative protein abundance, fold induction compared to control
0
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0.1 nM CP
1 nM CP
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100 nM CP
0.1 nM DCF
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10 nM DCF
UP
EFF
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R1
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R4
C
Smyhc1
relative protein abundance, fold induction compared to control
0
1
2
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4
5
0.1 nM CP
1 nM CP
10 nM CP
100 nM CP
0.1 nM DCF
1 nM DCF
10 nM DCF
UP
EFF
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R1
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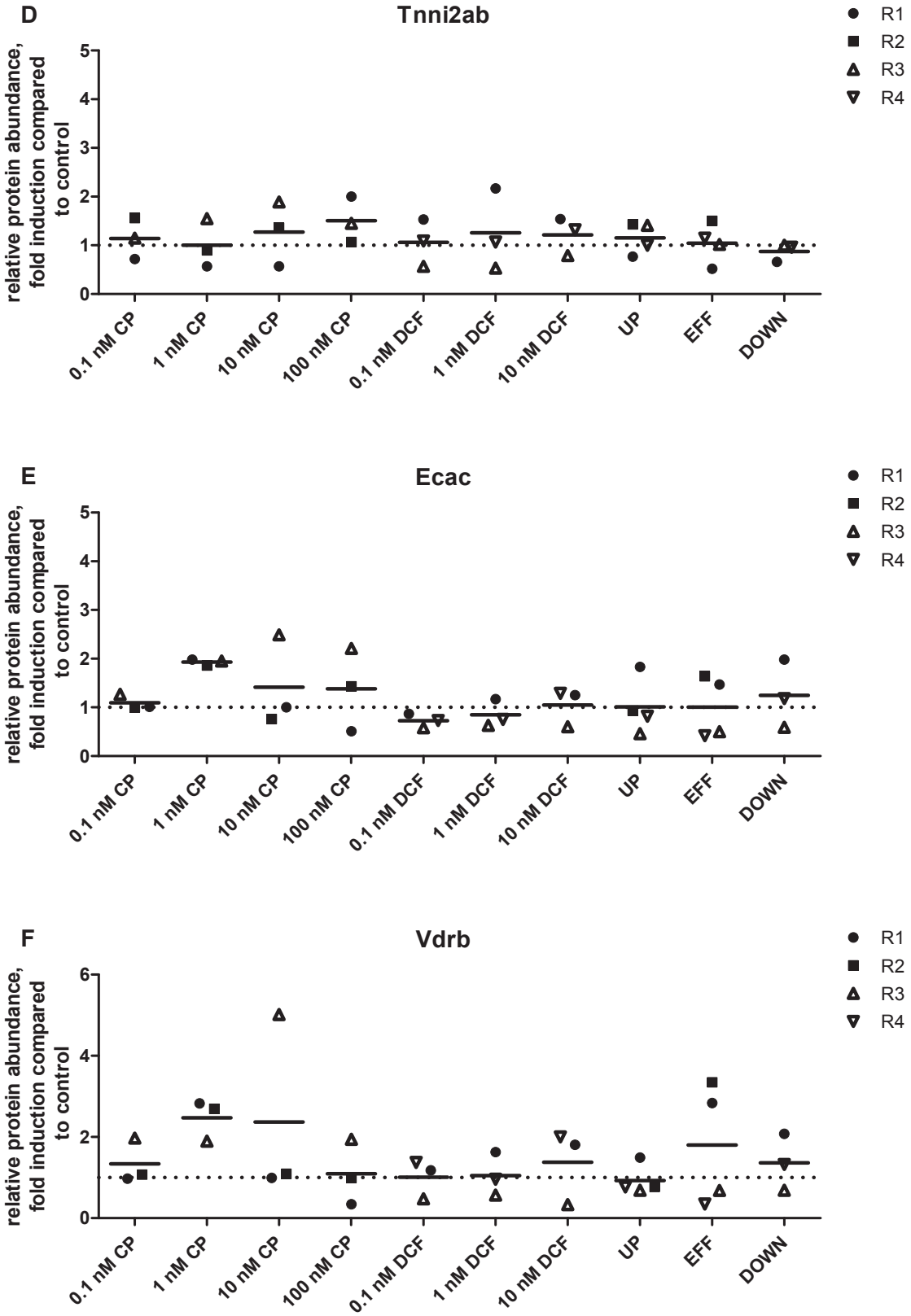
R3
R4


D
Tnni2ab
R1
R2
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R4
relative protein abundance, fold induction compared to control
0
1
2
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0.1 nM CP
1 nM CP
10 nM CP
100 nM CP
0.1 nM DCF
1 nM DCF
10 nM DCF
UP
EFF
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E
Ecac
R1
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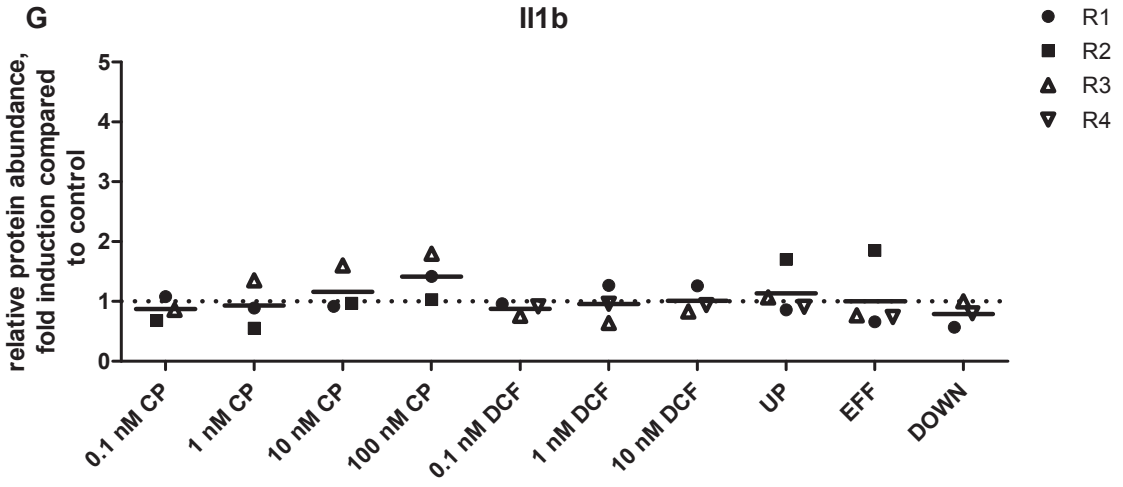
R4
relative protein abundance, fold induction compared to control
0
1
2
3
4
5
0.1 nM CP
1 nM CP
10 nM CP
100 nM CP
0.1 nM DCF
1 nM DCF
10 nM DCF
UP
EFF
DOWN
F
Vdrb
R1
R2
R3
R4
relative protein abundance, fold induction compared to control
0
2
4
6
0.1 nM CP
1 nM CP
10 nM CP
100 nM CP
0.1 nM DCF
1 nM DCF
10 nM DCF
UP
EFF
DOWN



SI Figure 4.1: Gene expression patterns of detectable GC-action related genes on the protein level. *Embryos were exposed to 0.1, 1, 10 and 100 nM clobetasol propionate (CP); 0.1, 1 and 10 nM diclofenac (DCF) or the filtered environmental grab water until 4 days post fertilization. The results of all biological replicates are plotted as fold changes compared to control. The control (fold change=1) is marked by a dashed line. The names of the examined proteins are indicated above the graphs. N=3 independent experiments for the CP and DCF exposures, and N=4 over the course of two weeks using the same samples for the environmental exposures (UP: upstream, EFF: WWTP effluent, DOWN: downstream). The results were analyzed by one-way ANOVA followed by Dunnett's test.*

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Chapter 5:

The response of the zebrafish embryo metabolome to clobetasol propionate

The analysis of metabolic changes provides a snapshot of the metabolites, the products of chemical processes resulting from specific cellular reaction cascades. Metabolites are also more conserved across species than gene products, which might allow for easier species comparison. Thus, to obtain this additional level of information, a metabolomics study was conducted with zebrafish embryos exposed to CP from 4 to 5 days post fertilization (dpf) using global and targeted metabolomics techniques. The protocols were initially developed for the aquatic snail *Lymnaea stagnalis* by another research group. Zebrafish embryo samples were analyzed in this partner laboratory as part of a secondment. The analysis by the global approach showed differences of the CP-exposed embryos compared to the control group. The data across the biological replicates was fluctuating. Accordingly, only four out of the 87 identified compounds showed significant differences: lysine and nicotinamide were increased while choline and hypoxanthine showed decreased levels after ≥1 nM CP exposure. These results may indicate increased protein degradation (lysine); a higher need of oxidation-reduction cofactors in the organism and anti-inflammatory effects (nicotinamide); an impact on the cholinergic neurotransmitter pathway (choline); and a decreased nucleic acid and ATP catabolism and possible ant-inflammatory effects (hypoxanthine). The targeted approach was focusing on neurotransmitters: tyrosine (a dopamine, epinephrine, and norepinephrine precursor) and tryptophan (a serotonin precursor) were significantly up-regulated after ≥1 nM CP exposure. This may indicate the activation of these neurotransmitter pathways and result in behavioral changes.

5.1 Introduction

The molecular changes induced in aquatic organisms by exposure to environmental pollutants can not only be assessed on the physiological or gene expression levels but also by analyzing the metabolome or certain groups of metabolites. The metabolome directly reflects the actual state of an organism at a given time point. This differs from transcriptomics and proteomics which describe activated processes leading to changes in gene expression levels both on the transcriptome and proteome level, and posttranslational modifications. As a consequence, environmental metabolomics has become a growing field with a lot of potential to reveal the molecular mechanisms behind the harmful effects of anthropogenic compounds on non-target organisms (Bundy et al., 2009; Tufi et al., 2015c, Elie et al., 2015; Tufi et al., 2016a; Tufi et al., 2016b; Viant et al., 2003). The analysis of the metabolome may also provide candidate biomarkers that can be used for monitoring and identifying compounds with similar effects using effect-directed analysis (EDA).

As suggested by their name, glucocorticoids (GCs) have an effect on glucose metabolism. One of the primary functions of cortisol is to raise blood sugar levels by enhancing gluconeogenesis in the liver. Indeed, GC-induced hyperglycemia and the resulting development of diabetes is a known phenomenon in humans (Clore and Thurby-Hay, 2009; Ogawa et al., 1992; Uzu et al., 2007). Furthermore, it was also found that low nanomolar GC exposure increases glucose levels in fish (Carney Almroth et al., 2014; Kugathas et al., 2013; Kugathas and Sumpter, 2011; Margiotta-Casaluci et al., 2016; summarized in Chapter 1, Table 1.1). Hence, exposure to CP may cause high blood sugar levels in zebrafish (*Danio rerio*) embryos too. The glucose levels of embryos were examined after different exposure durations, i.e. from 0 to 5, from 3 to 5 and from 4 to 5 days post fertilization (dpf). Although the main molecular components of the HPI axis appear to be developed by 48 hours post fertilization (hpf) (Alsop and Vijayan, 2009), stress-induced cortisol synthesis in zebrafish can only be detected at 72 hpf (osmotic stress, Alderman and Bernier, 2009; swirling stress Nesan and Vijayan, 2016) or 97 hpf (handling stress, Alsop and Vijayan, 2009). Thus, by the time the shorter exposures with CP started, the HPI axis was formed and functional. Therefore, our first aim was to examine the effects of GCs on the glucose levels after different exposure durations.

Using liquid chromatography coupled to mass spectrometry (LC-MS) based techniques one can screen for hundreds of metabolites simultaneously. Furthermore, metabolites are more conserved across species than gene products, and thus methods developed for one species can be used in other species as well. For instance, a PhD project within EDA-EMERGE investigated the effects of neonicotinoids on the aquatic snail *Lymnaea stagnalis* using metabolomics (Tufi et al., 2015c). For this, Tufi and coworkers developed global (Tufi et al.,

2015b) and targeted metabolomics approaches for the analysis of neurotransmitters and their precursors metabolites (Tufi et al., 2015a). The same targeted approach could also be used to monitor changes in neurotransmitter profiles during early zebrafish development and after pesticide exposure (Tufi et al., 2016a). GCs have been shown to cause anxiety in zebrafish (Khor et al., 2013). This can be the result of an imbalance in neurotransmission. Moreover, interactions between GC and serotonin signaling have been demonstrated with this fish species (Griffiths et al., 2012). Thus, the effects of CP exposure in zebrafish embryos could also be assessed with the above mentioned metabolomics platforms.

Due to time limitations, we could not examine all the above described exposure periods (i. e. 0 to 5, 3 to 5 and 4 to 5 dpf, respectively). We chose to use a short term CP exposure from 4 to 5 dpf, as the HPI axis is fully developed and functional by this time. Furthermore it is easier to carry out experiments, as the embryos are already hatched. This scenario would provide a rapid bioassay with only one day exposures. Therefore, we aimed to examine the metabolome of zebrafish embryos after a short-term CP exposure from 4 to 5 dpf, using the methods developed by Tufi and coworkers.

5.2 Materials and methods

5.2.1 Zebrafish husbandry and embryo collection

Wild type embryos with mixed genetic background from WiK (Max Planck Institute for Developmental Biology, Tübingen, Germany) and Qualipet (petshop, Switzerland) were used for the glucose level measurements. A wild type strain (OBI, Helmholtz Centre for Environmental Research established from OBI hardware store, Leipzig, Germany) was used for the metabolomics study. Zebrafish were maintained according to recommended procedures (Nüsslein-Volhard and Dahm, 2002). Adult zebrafish were kept in 60 L aquaria with dilution water (294 mg/L $CaCl_2 \cdot 2\ H_2O$, 123.2 mg/L $MgSO_4 \cdot 7\ H_2O$, 64.74 mg/L $NaHCO_3$, and 5.75 mg/L KCl; prepared according to OECD test guideline 236) using a 14/10 h light/dark cycle at 28 °C. The embryo collecting dishes were placed in the aquaria in the afternoon the day before collection. The embryos were collected 45 min after the light turned on in the morning, washed, and kept in dilution water. Next, the fertilized embryos were selected and placed in the pre-soaked petri dishes when the exposure started from 0 dpf (glucose level assay, long exposure) or into petri dishes with dilution water (shorter exposure for glucose level assay, metabolomics), depending on the experiment type and duration.

5.2.2 Zebrafish embryo exposures for glucose level assay

In the exposures for glucose level measurements, CP was first dissolved in DMSO and then spiked into the dilution water. Final DMSO concentration in these experiments was 10 µL/L, i.e. 0.001 % v/v. Embryos were exposed from 0 to 5 dpf, from 3 to 5 dpf and from 4 to 5 dpf. The embryos were kept in small petri dishes (d=60 mm) in 15 mL dilution water, 20-25 embryos per dish. Only 70 % of the exposure solutions were changed every day during the experiments in order to avoid stressing the embryos. After the end of exposures, embryos were anaesthetized by immersing them in ice-cold dilution water, and collected into 1.5 mL Eppendorf tubes. After removing excess liquid, they were immediately snap-frozen in liquid N_2.

5.2.3 Glucose level assay

Glucose levels in the embryos were determined after different exposure durations according to a protocol previously described (Jurczyk et al., 2011). The protocol is based on a glucose colorimetric/fluorimetric assay kit from BioVision (Milpitas, CA, USA) which measures free glucose. Samples were thawn in the presence of 8 µL/embryo assay buffer, then homogenized (LABSONIC® M, Sartorius Stedim Biotech GmbH, Germany; parameters: cycle 1, amplitude 100%, 3x10 sec, with 20-30 sec breaks, all on ice). Lysates were cleared by centrifugation for 10 min at 4 ^{0}C and 13,000 rpm. Then they were transferred into new 1.5 mL Eppendorf tubes and stored at -80 ^{0}C until analysis.

Before analysis, samples were gently thawed on ice. Then, the reactions were started on ice in black, flat bottom 96-well plates (Greiner). To measure glucose, 6 µL sample was added to 44 µL assay buffer. To this, 50 µL reaction mix was added containing 0.4 µL glucose probe (Amplex Red) and 1 µL enzyme solution (glucose oxidase, horseradish peroxidase). Standard curves were generated with the provided glucose standard solution according to the manual of the kit. Reactions were incubated for 30 min at 37 ^{0}C in the dark. Fluorescence (excitation 535 nm, emission 590 nm) was measured using a TECAN infinite M200 spectrophotometer (TECAN, Switzerland).

The measured values were corrected by subtracting fluorescence values of control reactions without embryo extracts. Glucose levels were calculated from the standard curves. Each sample was measured in triplicate.

5.2.4 Zebrafish embryo exposures for metabolomics

To perform exposures for the global and targeted metabolomics analyses, approximately 0.5-1 mg CP (Sigma-Aldrich, Switzerland) was dissolved in one liter of dilution water and the concentration was measured by a UV-Vis spectrophotometer (Cary 100, Agilent

Technologies, Switzerland) at 240 nm wavelength. The stock solution was kept at 4 °C up to one week. Embryos were exposed to 0, 0.1, 1 and 10 nM CP from 4 to 5 dpf at 28 °C using a 14/10 h light/dark cycle. Embryos were kept in big petri dishes (d=94 mm), with 40 mL solution and 30-40 embryos per dish. After the end of exposures, the embryos were anaesthetized by immersing them in ice-cold dilution water, and collected into 2 mL Precellys tubes with 1.4 mm ceramic beads (zirconium oxide) (CK 14, Bertin Technologies, France). After removing excess liquid, they were immediately snap-frozen in liquid N_2.

5.2.5 Sample preparation for metabolomics

The sample extraction for the global and targeted metabolomics analyses were carried out as described previously (Tufi et al., 2016a). The frozen samples were sent by a courier service on dry ice to the Institute of Environmental Studies (IVM), Vrije Universiteit Amsterdam, the Netherlands. 400 µL ice-cold Milli-Q water was added to the samples immediately after having taken them out of the -80 ^{0}C freezer and the samples were homogenized with the tissue homogenizer Precellys24 Dual device (Bertin Technologies, France) operated at 6500 rpm for two cycles of 10 s with a 15 s break between the cycles. 15 µL homogenate was taken and transferred into 2 mL Eppendorf tubes and stored at -80 ^{0}C until protein content measurement with the Bradford assay using the BioRad reagent (BioRad, USA). The remaining homogenates were extracted by adding 325 µL ice-cold MeOH and 75 µL ice-cold stable isotope-labeled internal standards in MeOH. The samples were homogenized again in the tissue homogenizer with the same program. The extracts were transferred into 2 mL Eppendorf tubes, and the Precellys tubes were rinsed twice with 50 µL MeOH/H_2O (70:30 v/v). An additional cooling step of 10 min on ice facilitated protein precipitation. The extracts were then centrifuged for 5 min at 4 ^{0}C at 17,000 rpm (Heraeus Biofuge stratos, Heraeus Instruments, Germany). The hydrophilic fractions (supernatants) were dried in a Centrivap concentrator (Labcono Co., MO, USA) for 240 min at 20 ^{0}C. The dried samples were reconstituted in 100 µL ACN/H_2O (90:10 v/v) and centrifuged for 5 min at 4 ^{0}C at 17,000 rpm, and then the samples were transferred into autosampler vials.

5.2.6 Global metabolomics

A cross-platform global metabolomics analysis of the hydrophilic fraction of extracts was used according to Tufi et al., 2015b. The samples were analyzed with an Agilent 1200 HPLC system (Agilent, USA) coupled to a high-resolution time-of-flight MS (MicroToF II, Bruker Daltonics, Germany). The HILIC separation was achieved using a Waters XBridge Amide column (150×2.1 mm, 3.5 µm particle size) equipped with an XBridge Amide guard column (10×2.1 mm, 3.5 µm particle size). Mobile phase A consisted of ACN/H_2O 30:70 v/v NH_4HCO_2 10 mM, and B of ACN/H_2O 95:5 v/v NH_4HCO_2 10 mM. During gradient elution B

was increased from 0 to 100% in 25 min. The flow rate was set to 0.25 mL/min, the column temperature to 30 ^{0}C and 5 µL sample was injected. The electrospray ionization (ESI) MS source was operating in positive and negative mode scanning from 50 to 1000 m/z with a rate of 1 Hz. The capillary was set to ±2500 V, the end set plate to ±500 V, the N_2 nebulizer gas at 4 bar, and the drying gas at 8 L/min at 200 ^{0}C. Internal calibration was conducted by infusing a solution of 5 mM sodium hydroxide in the sheath liquid of 0.2% formic acid in water/isopropanol 1:1 (v/v), and the resulting sodium formate clusters were used for mass calibration. This calibration solution was injected at the beginning of each run, allowing recalibration of each chromatogram. The stability of the instrumental performance was checked by injection of hydrophilic metabolite standards after every 7th sample.

5.2.7 Analysis of global metabolomics data

The data analysis was carried out according to Tufi et al., 2015b and Tufi et al., 2015c. The chromatograms were analyzed with the software DataAnalysis 4.0 (Bruker Daltonics, Germany). First, the spectra were calibrated using the internal calibration signals (sodium formate clusters). Tufi and coworkers analyzed >500 standards from the MS Metabolite Library of Standards (MSMLS; IROA Technologies, MI, USA) to determine their retention times and masses. The chromatograms were batch-processed with the software Compass PathwayScreener (Bruker Daltonics) to match features found in the samples with metabolites in the MSMLS library. The results were exported, and the metabolites that were detected in all the samples selected. The areas of these metabolites were summed up for each sample and all areas normalized to this sum. The normalized data was statistically evaluated by two-way ANOVA followed by Bonferroni posttests (GraphPad Prism 5.0).

The mass calibrated chromatograms were processed with the Find Molecular Features (FMF) algorithm of DataAnalysis 4.0, that combines isotopes, charge states, adducts or neutral losses belonging to the same compound into one feature. The selected settings were: S/N threshold=5, correlation coefficient threshold=0.7, minimum compound length: 8 spectra, smoothing width=2. Each feature is defined by retention time, m/z value and intensity. The molecular features were processed with the ProfileAnalysis 2.1 software (Bruker Daltonics, Germany). The intensity values were normalized to the largest bucket value in each sample in order to compare different samples. The variables of each bucket table were normalized to the total metabolite content in Microsoft Excel. The generated data matrix was imported into SIMCA-P+ 14.0 (Umetrics, Umeå, Sweden) for multivariate data analysis (MVDA). After Pareto scaling, principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), and orthogonal partial least squares discriminant analysis (OPLS-DA) were performed.

5.2.8 Targeted metabolomics

The targeted metabolomics analyses measuring neurotransmitters, their precursors and metabolites were carried out according to Tufi et al., 2016a. Briefly, a 1260 Infinity Binary LC system from Agilent Technologies coupled to a 6400 series triple quadrupole mass spectrometer from Agilent equipped with an ESI source was used.

Table 5.1: Overview of neurotransmitters and their precursors and metabolites monitored. *Compounds in bold were quantifiable in zebrafish embryos in the study conducted by Tufi and coworkers (adapted from Tufi et al., 2016a). LOQ: limit of quantitation, NVA: no value available.*

Neurotransmitter system	Compound	Function	LOQ (ng/mL)
histaminergic	histamine	neurotransmitter	76
	histidine	precursor	0.9
glutaminergic/ GABAnergic	**γ-aminobutyric acid (GABA)**	neurotransmitter	58
	glutamic acid	neurotransmitter	7.4
	glutamine	precursor/metabolite	4.5
cholinergic	**acetylcholine**	neurotransmitter	0.1
	choline	precursor/metabolite	1
serotonergic	**serotonin**	neurotransmitter	0.3
	5-hydroxy-L-tryptophan	precursor	11
	tryptophan	precursor	1.6
	5-hydroxyindoleacetic acid (5-HIAA)	metabolite	12
dopaminergic/ adrenergic	norepinephrine	neurotransmitter	40
	normetanephrine	metabolite norepinephrine	0.9
	epinephrine	neurotransmitter	17
	dopamine	neurotransmitter	17
	3,4-dihydroxyphenylalanine (DOPA)	precursor dopamine, norepinephrine, epinephrine	58
	3-methoxytyramine (3-MT)	metabolite dopamine	NVA
	phenylalanine	precursor dopamine, norepinephrine, epinephrine	0.1
	tyrosine	precursor dopamine, norepinephrine, epinephrine	25

Separation was performed on a zwitterionic ZIC-cHILIC column (150×2.1 mm, 3 µm particle size) equipped with a ZIC-cHILIC guard column (20×2.1 mm, 5 µm particle size) from Merck-Sequant (Germany). Mobile phase A consisted of 100% Milli-Q H_2O and B of 90:10 v/v ACN/H_2O, both buffered with NH_4HCO_2. The MS source was operated in positive mode. Selected reaction monitoring (SRM) data was acquired and quantified using MassHunter (Agilent Technologies). The detected compounds were normalized to the sample protein content. The results were analyzed with one-way ANOVA followed by Dunnett's test (GraphPad Prism 5.0). An overview of the monitored metabolites is given in **Table 5.1** (adapted from Tufi et al., 2016a).

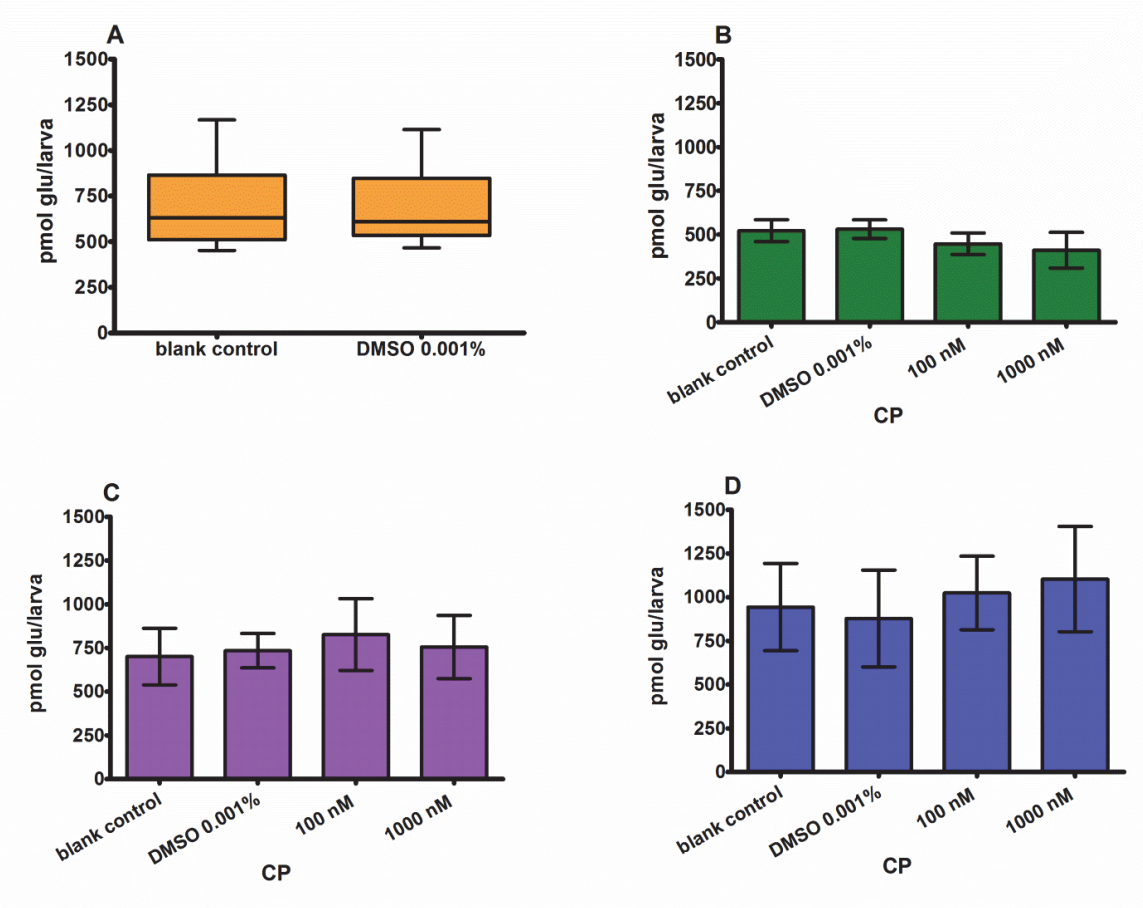


Figure 5.3.: CP effects on glucose levels in zebrafish embryos after different exposure durations. ***A:*** *comparison of blank control and 0.001 % DMSO over all of the exposures carried out, N=10 independent experiments.* ***B:*** *embryos were exposed from 0-5 days post fertilization (dpf), N=4 independent experiments, except for 1000 nM, where N=3.* ***C:*** *embryos were exposed from 3-5 dpf, N=3 independent experiments.* ***D:*** *embryos were exposed from 4-5 dpf, N=3 independent experiments. The horizontal line in the box plots (**A**) demonstrates the mean of the values, the error bars represent SD. The bar graphs were plotted as mean ± SD. The data was analyzed with one-way ANOVA, but no statistical significance was found.*

5.3 Results

5.3.1 CP effects on glucose levels after different exposure durations

The glucose levels in blank control and solvent controls did not show any differences (**Fig. 5.3A**), thus DMSO did not regulate glucose levels in the embryos. Furthermore, there were no significant differences between the controls and CP-exposed embryos, neither after long-term, i.e. from 0 to 5 dpf exposures (**Fig. 5.3B**), nor shorter exposures: from 3 to 5 dpf or from 4 to 5 dpf (**Fig. 5.3C and D**).

5.3.2 CP effects on the metabolome: non-targeted metabolomics

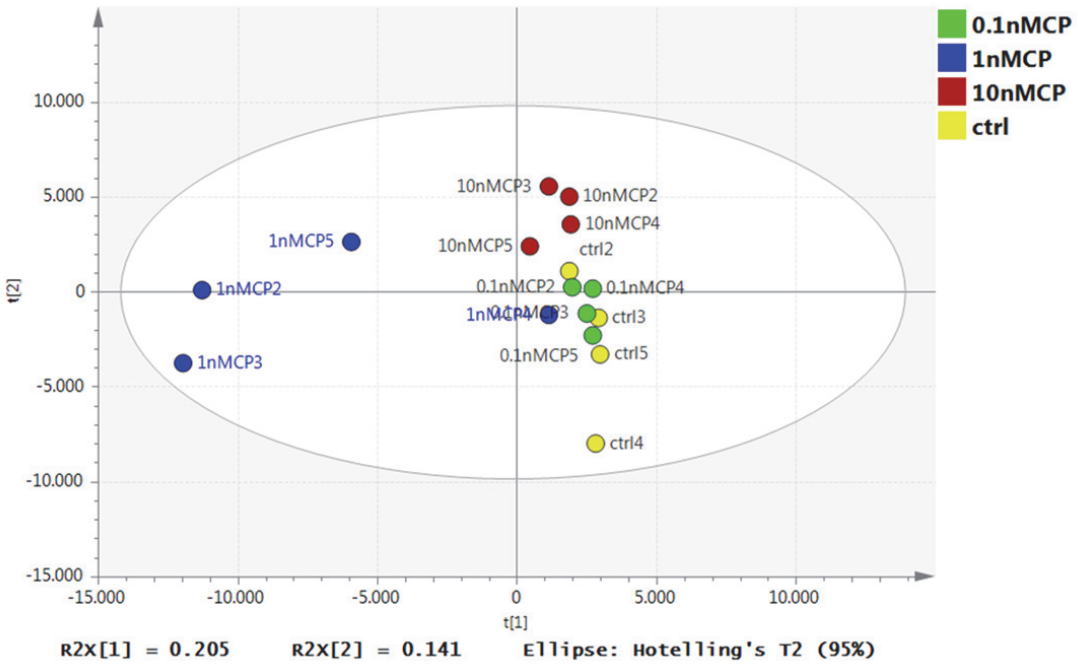
The batch analysis carried out with the Compass PathwayScreener software identified 257 metabolites from the MSMLS in the zebrafish embryo extracts. However, several metabolites were only detected in few samples, preventing a statistical analysis. Four biological replicates were analyzed, but some metabolites that were present in all the other samples were not identified in the control sample of the 4th replicate. Thus, the whole replicate was excluded from the statistical analysis of the identified metabolites. At the end, 87 metabolites were evaluated. The two-way ANOVA with Bonferroni post hoc analysis of the remaining three replicates resulted in a few compounds that were significantly regulated by CP exposure (**Table 5.2.**)

Table 5.2: Significantly regulated metabolites after CP exposure. *N=3 independent expriments (* p<0.05, ** p< 0.01, *** p<0.001, two-way ANOVA with Bonferroni post hoc analysis).*

ctrl vs CP exposure	significantly regulated metabolites
ctrl vs 0.1 nM	no metabolites
ctrl vs 1 nM	choline down (*), hypoxanthine down (*), lysine up (***), nicotinamide up (**)
ctrl vs 10 nM	hypoxanthine down (**)

The MVDA analysis of the molecular features found by the FMF algorithm did not reveal significant differences between the control and CP-exposed zebrafish embryos with OPLS-DA. The PLS-DA score plots (**Fig. 5.1**) show grouping of the different treatments on the data acquired that is more pronounced in the negative ion mode.

A



B

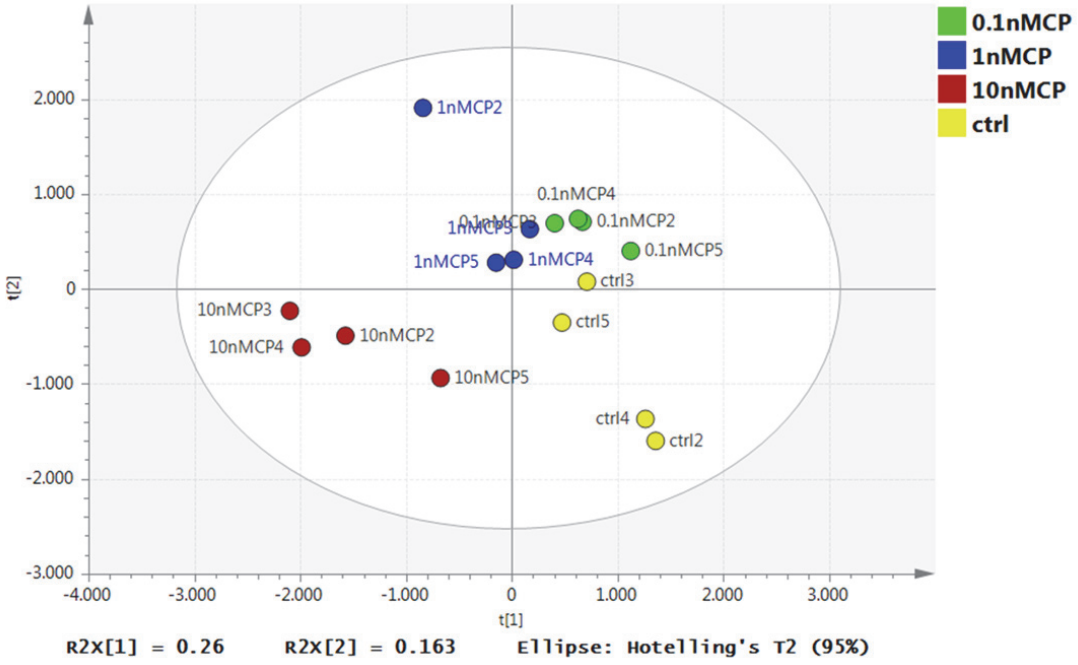


Figure 5.1: Partial least square discriminant analysis (PLS-DA) score plots of blank control and clobetasol propionate (CP)-exposed embryos. ***A:*** *positive ion mode and* ***B:*** *negative ion mode. N=4 independent experiements.*

5.3.3 CP effects on the metabolome: targeted metabolomics (neurotransmitters)

All the compounds that had been quantified in zebrafish embryos in the study by Tufi and coworkers (**Table 5.1.**) could be detected in this study as well. However, acetylcholine, glutamic acid and epinephrine were below LOQ levels. Among the neurotransmitters, their precursors and metabolites, the levels of tryptophan and tyrosine showed significant alterations caused by CP exposure (**Fig. 5.2.**).

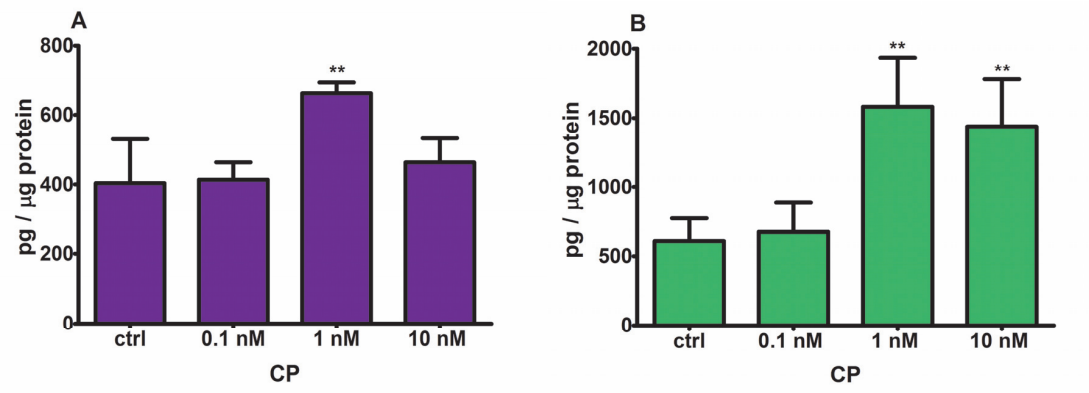


Figure 5.2.: Tryptophan (A) and tyrosine (B) levels after CP exposure. *N=4 independent experiments, except for 1 nM group, where N=3. Results are plotted as mean ± SD. The results were analyzed by one-way ANOVA followed by Dunnett's test, **p<0.01*

5.4 Discussion

We found no regulation of glucose levels after exposure to CP. However, glucose levels of transgenic zebrafish embryos have been found to be elevated after exposure from 4 to 6 dpf to 10 µM GCs (budenoside, beclomethasone, dexamethasone) (Gut et al., 2013), but then our highest concentration was ten times lower at 1 µM. On the other hand studies with adult fish found glucose level elevation after low nanomolar GC exposures (Carney Almroth et al., 2014; Kugathas et al., 2013; Kugathas and Sumpter, 2011; Margiotta-Casaluci et al., 2016). Because the zebrafish embryos were 5 dpf old, and thus their yolk sac mostly consumed and external feeding expected to start, our observation may be due to the fact that the embryos were in their final fasting state. Gut and coworkers also measured the glucose levels in control embryos from 3 to 7 dpf without feeding, to examine the fasting state of the embryos. They found that the glucose levels peaked at 5 dpf, at the time we sampled the embryos for our analyses. Therefore, it might be that in this condition the embryos could not further increase the glucose levels. Thus, choosing the time point for analysis appropriately is crucial, because we might have found elevated glucose levels – as was found in adult fish – at 4 dpf, when the embryos are not in a fasting state yet.

The zebrafish embryo exposures to CP were carried out using 0.001% DMSO as a solvent vehicle. In this case, the solvent control and blank control results were not significantly different. However, since we have found that using this DMSO concentration can interfere with the bacterial lipopolysaccharide (LPS) challenge assay (Chapter 3) we decided not to use any DMSO in our metabolomics study.

We examined the response of the zebrafish embryo metabolome after CP exposure. Non-targeted and targeted metabolomics both identified only a few compounds that were significantly regulated. Zebrafish embryos exposed to 0.1 nM CP, an environmentally relevant concentration, showed no significant regulation of the identified compounds obtained from the non-targeted metabolomics. There were also no significant changes in the compounds related to neurotransmitter metabolism quantified by targeted metabolomics. The lack of statistically significant differences in response to 0.1 nM CP may be due to the short exposure duration. Hence the effects of longer exposure durations should be assessed before concluding on the overall impact of this CP concentration in general.

After 1 nM CP exposure, lysine and nicotinamide had significantly increased levels. Lysine is an essential amino acid that is the building block of many proteins, thus its increased levels may indicate a generally induced protein synthesis and/or degradation. Nicotinamide is the constituent of nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) which are two essential cofactors involved in oxidation-reduction reactions. Nicotinamide also exerts anti-inflammatory properties in several human cell types, in part via activation of the transcription factor Forkhead box O3 (FoxO3) (Lappas and Permezel, 2011). FoxO3 transcription is activated upon GR activation (Lutzner et al., 2012). Lappas and Permezel showed that nicotinamide significantly suppressed LPS-induced pro-inflammatory cytokines tumor necrosis factor alpha (TNFα) interleukin-6 (IL-6), the chemokine IL-8, the cyclooxygenase (COX)-2 enzyme, and the prostaglandins PGE_2, $PGF_2\alpha$ and in human placenta. In Chapter 3 I demonstrated that the transcripts of *anxa1b*, a key regulatory enzyme of the arachidonic acid synthesis that involves COX-2 and prostaglandins (Chapter 4, Fig. 4.1), and *il8* were significantly down-regulated in embryos exposed to CP from 0 to 4 dpf. Furthermore, the suppression of IL-8 by nicotinamide was shown to be due to inhibition of the NFκB and MAPK pro-inflammatory pathways in HaCaT cells (Grange et al., 2009). The down-regulation of these pro-inflammatory pathways was also demonstrated in Chapter 3. Thus, it might be that the observed anti-inflammatory effects in zebrafish embryos after 4 days CP exposure were also due to an increased level of nicotinamide. However, this needs further confirmation.

Choline was decreased by 1nM CP exposure compared to control. Choline is the precursor of the neurotransmitter acetylcholine. One of the most important roles of acetylcholine is the

activation of skeletal muscles through the neuromuscular junctions. Acetylcholine was monitored and detected in the targeted metabolomics method. However the detected concentrations were below LOQ levels. Thus, we cannot correlate the choline and acetylcholine levels. The decrease in the precursor level can result from the induction of acetylcholine. In this case choline acetyltransferase, the enzyme converting choline into acetylcholine would be up-regulated, because then the amount of choline is decreasing due to the enhanced activity of the enzyme. However, the decrease in choline levels can also mean the decrease of the neurotransmitter too, when the whole cascade of acetylcholine biosynthesis would be inhibited. The GC dexamethasone was demonstrated to inhibit the development of cholinergic neurons in the forebrain of postnatal rats after prolonged exposure (Hu et al., 1996; Kreider et al., 2005). Thus, the decreased level of choline may mean a similar impairment of the development of cholinergic neurons in zebrafish embryo. This needs further investigation.

Choline was also monitored and detected with the targeted method, where interestingly no significant difference was observed with any of the CP exposure concentrations (**Fig. SI 5.1A**). This may be because the two datasets were normalized with different values. The non-targeted data were normalized to the sum of the areas of 87 metabolites that were detected in all the samples, because we hypothesized that this would be an appropriate estimation of the total abundance of metabolites in the samples. The neurotransmitter data was normalized to the protein content of the samples, to take the amount of tissue into account. To check this, we re-evaluated the global data using protein content normalization, and in this case indeed the choline concentration after 1 nM CP exposure was not significantly different from the control. As metabolomics analysis is still a relatively young field, the optimal procedures for normalization and interpretation of the data are still being actively developed and debated (Wu and Li, 2016).

Hypoxanthine was significantly decreased after both 1 nM and 10 nM CP exposures. Hypoxanthine, a by-product of ATP catabolism, has been shown to be a biomarker of cardiac ischemia in humans (Farthing et al., 2015). Hypoxanthine is also the product of purine metabolism (Feng and Yeung, 2000). Thus, the significantly decreased amount of hypoxanthine after CP exposure may be an indication of reduced ATP catabolism and thus elevated ATP levels or generally a decreased purine metabolism. Elevation in hypoxanthine levels were observed during endotoxin shock in mammals (Jabs et al., 1995; Oldner et al., 1999). This is what we were mimicking with the LPS challenge assay in Chapter 3. Moreover, uric acid, a product of hypoxanthine is also an endogenous danger signal in inflammation (Ghaemi-Oskouie and Shi, 2011). Thus, the reduced level of hypoxanthine detected after CP exposure may also support our previous observation that CP suppresses the inflammatory response in zebrafish embryos.

The targeted metabolomics method showed significant elevation of tryptophan after 1 nM, and tyrosine after both 1 and 10 nM CP exposures. Chronic stress, and with that a constantly activated hypothalamus-pituitary-adrenal (HPA) axis and thus GR function on gene expression patterns have been hypothesized to be the main cause of depression and anxiety (Anacker et al., 2011). The deficit of the neurotransmitter serotonin has also been associated with depression in humans (Deakin, 1998). We did not detect any significant difference in serotonin levels after CP exposure in zebrafish embryos (**SI Figure 5.1B**). However, its precursor, tryptophan, was elevated in our study. Tryptophan hydroxylase, the enzyme that catalyzes the synthesis of serotonin from tryptophan, has been shown to be regulated by GC administration in adrenalectomized rats (Clark and Russo, 1997). However, the modulation was tissue-specific, showing down-regulation in the brain, which was correlating with decreased serotonin levels. Tryptophan is also the precursor of nicotinamide, thus the elevation of both tryptophan and nicotinamide may imply the necessity of forming more NAD and NADPH, and the above described anti-inflammatory actions of nicotinamide. Also, impaired tryptophan catabolism is linked to inflammation and thus to depression (Anderson et al., 2012; Romani et al., 2008)

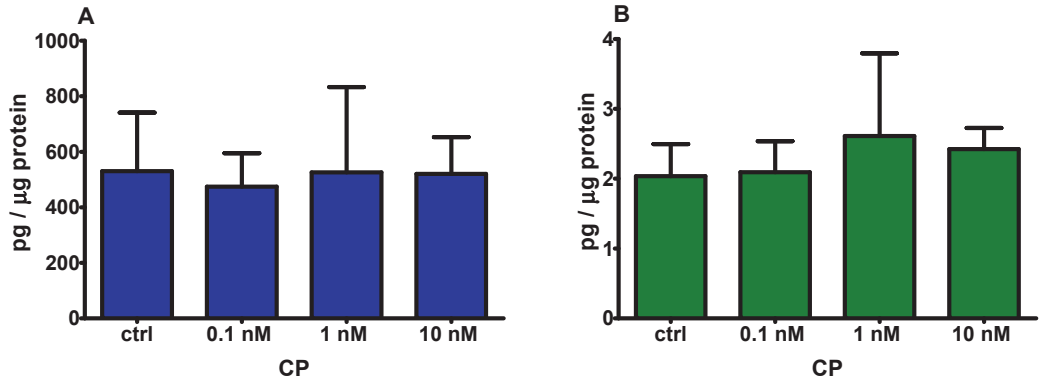
Tyrosine is the precursor of dopamine, norepinephrine and epinephrine. Unfortunately, neither of these neurotransmitters could be detected in the zebrafish embryo extracts. Thus, we cannot say anything regarding which neurotransmitters, if any, were altered due to the increase of tyrosine. Tyrosine aminotransferase, an important enzyme in gluconeogenesis is known to be activated by GCs. However, as we did not find any elevated glucose levels, this pathway seems not relevant for our results.

Both tyrosine and tryptophan are also used as building blocks for proteins, thus the observed increase may be related to general protein synthesis and/or degradation, as was hypothesized for the elevated lysine levels too.

Our study showed that only a few metabolites were significantly regulated after a one-day CP exposure at the time when the HPI axis is fully functional in zebrafish embryos. Nonetheless, some potential effects of CP could already be seen, such as the down-regulated hypoxanthine levels and the up-regulated tryptophan-nicotinamide metabolism, both of which may indicate the suppression of the inflammatory response in zebrafish embryos. However, to confirm and expand the preliminary findings reported in this chapter, the metabolomics analyses should be repeated after longer CP exposures and possibly different sampling times.

Contributions: I conducted all the zebrafish embryo exposures used for this chapter. I optimized the glucose level assay, and carried out the measurements. I did the sample preparation for the metabolomics analyses at IVM, VU, Amsterdam with the great help of Sara Tufi, and she ran the non-targeted and targeted metabolomics LC-MS analyses. Then, I carried out the data evaluation for both approaches with her help.

5.5 Supplementary information



SI Figure 5.1: The level of choline (A) and serotonin (B) after CP exposure determined by the targeted method. *N=4 independent experiments, except for 1 nM group, where N=3. Results are plotted as mean ± SD. The data was analyzed with one-way ANOVA, but no statistical significance was found.*

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Chapter 6:
Conclusions and outlook

The aim of this thesis was to assess the effects of environmentally relevant concentrations of synthetic glucocorticoids (GCs) on zebrafish embryos, focusing on the inflammatory response and molecular mechanisms of GC action by looking at mRNA and protein expression levels and metabolite abundance.

The main outcomes from this dissertation can be summarized as follows:

- The GCs clobetasol propionate (CP) and fluticasone propionate were found to be the responsible compounds for most of the glucocorticoid receptor (GR) activity detected in the effluent of a wastewater treatment plant (WWTP) in Switzerland.
- The inflammatory response of zebrafish embryos was significantly reduced by exposure to environmentally relevant concentrations of CP. As this may make the organism more susceptible to pathogens, GCs present in the aquatic environment appear to have the potential to reduce the survival chances of fish.
- Several inflammatory response or GC action-related genes in zebrafish embryos on the mRNA and protein level were shown to be regulated by either CP or the non-steroidal anti-inflammatory drug (NSAID) diclofenac (DCF), or both these compounds. Among the regulated genes, annexin a1b (*anxa1b*) is proposed as a candidate biomarker of anti-inflammatory effects.
- The metabolomics study revealed choline, hypoxanthine, lysine, nicotinamide, tryptophan and tyrosine to be regulated after a short CP exposure in zebrafish embryos. These results may support the immunosuppressive effects of CP in fish.

In the following part, the most important outcomes, conclusions and encountered difficulties with regard to the detection of environmental GCs and investigation of their effects in zebrafish embryos are discussed. The discussion of each considered aspect is concluded by perspectives for further research.

The higher-tiered effect-directed analysis (HT-EDA) study determined the contamination of Urtenen river waters impacted by a municipial WWTP by a mixture of endocrine disrupting chemicals (EDCs) through the combined use of passive sampling and mechanism-based bioassays. *In vitro* screening revealed the WWTP effluent discharge site as a source of GCs, estrogens and other contaminants. The fractionation of the crude extract and *in vitro* testing of the fractions showed a clear separation of estrogenic and glucocorticoid receptor-active chemicals. The chemical analysis targeting several GCs in the GR-active fractions found CP and fluticasone propionate to be responsible for most of the total GR activity in the sample.

However, 20% of the activity could not be accounted for. For the identification of the remaining GCs, non-target chemical analysis would have to be applied. Overall, Chapter 2 demonstrates that EDA can be successfully used to identify compounds that are responsible for specific biological effects.

The LPS challenge assay demonstrated for the first time a significantly reduced inflammatory response after exposure to environmentally relevant CP concentrations. Such an effect may make fish more susceptible to pathogens, reducing their survival chances. Therefore, the potential of GCs to affect the vulnerability of fish to various pathogens and other environmental stressors should be explored in the future. Additionally, it was noted that 0.001% dimethyl sulfoxide (DMSO) masked the immunosuppressive effect of CP. This finding calls for alternative dosing methods to assure reliable exposure concentrations. If chemical properties prohibit solubilization without an organic solvent, passive dosing has been proposed as alternative. In any case, confirmation of true exposure concentrations and, where applicable, reference to environmentally relevant concentrations are important to properly understand exposure and effect.

In order to understand the molecular mechanisms behind the observed immunosuppressive effects, the dynamics of ten inflammatory response and/or GC action-related genes were investigated. Six genes were regulated after CP exposure, four of which were part of the LPS-induced inflammatory pathway: the MAPK signaling (*p38, il8*) and NFκB pro-inflammatory (*IκBα)* pathways were down-regulated, while the general stress and the cytosolic GR complex component (*hsp70*) were up-regulated. These results indicate that the LPS-induced inflammatory cascade is conserved from mammals to fish. *Anxa1b* was the only gene that showed significant responses at the lowest effective concentration in the LPS challenge assay. However, based on the knowledge available from mammals, *anxa1b* is not directly involved in the LPS-induced response. Hence, the regulation of other inflammatory genes should be monitored using microarrays or RNA sequencing, in order to understand the complex molecular mechanism behind the anti-inflammatory effects of GCs in zebrafish embryos. Moreover, *anxa1b* would be an interesting gene to further explore for its specific functions in the zebrafish embryo, e.g. by functional genomics approaches.

Analyzing gene expression on the protein level gives additional information on the health state of the organism compared to the transcriptome, as proteins perform essential functions. To analyze if mRNA alterations elicited by the CP exposure also translate into a differential expression of proteins, an LC-MS/MS-based targeted proteomics technique was developed. We detected only 12 out of the selected 40 GC action-related proteins in zebrafish embryos. This is due to the fact that zebrafish embryo digests are extremely complex samples and detecting several proteins simultaneously is a big challenge. Protein expression was found to

be more variable than mRNA expression, and hence the differences between treatment and control groups appeared smaller. A higher significance could be obtained by increasing the number of replicates, and sensitivity could be improved by the use of affinity techniques. Yet, the latter technique requires specific antibodies that are still difficult to obtain for zebrafish.

In order to check GC specificity of the candidate genes, the NSAID DCF, which does not activate the GR, was used. Also, to check for robustness in a complex matrix, grab water samples containing GCs and DCF from the HT-EDA sites were collected and used for zebrafish embryo exposures. The analysis of inflammatory response-related genes in the LPS study provided 6 significantly regulated transcripts after CP exposure. Two of them (*cfos* and *anxa1b*) were responding to the environmental samples in a similar way as to CP and DCF. However, *cfos* is also used as a biomarker of neuronal activity and hence not specific enough. Thus, only *anxa1b* is a candidate biomarker for anti-inflammatory effects in zebrafish embryos. On the protein level, only the muscle protein Myhz2 was significantly regulated after DCF exposure, while IκBα was regulated after CP exposure on both the mRNA and protein level. None of these proteins showed regulation after environmental water exposures. Since Anxa1b could not be monitored on the protein level due to sensitivity issues, alternative techniques should be tested.

The analysis of metabolic changes provides a snapshot of the metabolome. We found that the glucose levels were not elevated following different durations of CP exposure. This may be caused by the fact that we sampled the embryos at 5 dpf, when the yolk sac has mostly been consumed. Thus, the embryos might have been in a fasting state, and hence the glucose levels could not increase. For this reason these experiments should be repeated at an earlier developmental phase, when the yolk sac is not yet fully consumed.

The metabolomics study revealed only a few metabolites that were significantly regulated after CP exposure from 4 to 5 dpf, at the time when the HPI axis is fully functional. Choline and hypoxanthine levels were decreased, while lysine and nicotinamide, tryptophan and tyrosine levels were increased. Regulation of hypoxanthine and tryptophan-nicotinamide levels may indicate the suppression of the inflammatory response in zebrafish embryos. We did encounter problems with the normalization of the various data sets that will need to be addressed in the future. To confirm and expand the preliminary metabolomics findings, the analyses should be repeated after longer CP exposures and possibly different sampling schemes.

In conclusion, this thesis offers an insight into the environmental presence of GCs in freshwaters and shows that GCs exert immunosuppressive effects in fish at environmentally relevant concentrations. Thus, the anti-inflammatory effects of GCs seem to be conserved from mammals to fish. The GCs detected in the environment may make fish more

susceptible to pathogens and thus potentially reduce their chance of survival. The response of the inflammation-related gene *anxa1b* to a GC and an NSAID was shown to be robust enough to be detectable in complex environmental samples. Hence it is proposed as a candidate biomarker for the detection of anti-inflammatory compounds in the aquatic environment.

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Curriculum vitae

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Education

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2010 – 2012	MSc in Biochemical Engineering, Budapest University of Technology and Economics, Faculty of Chemical Technology and Biotechnology; Mecsek Pharma Research, Development and Service Ltd., Budapest, Hungary Project: Mitochondrial analysis of the effects of herbal plants and BGP-15 during the development of insulin resistance (high fat diet mouse model)
2006 – 2010	BSc in Biochemical Engineering, Budapest University of Technology and Economics, Faculty of Chemical Technology and Biotechnology, National Center for Epidemiology, Microbiological Research Group, Budapest, Hungary Project: Analysis of epigenetic modifications at the lamin A promoter in cell lines carrying latent Epstein-Barr virus genomes
Laboratory skills	**LC-MS/MS** (Thermo Scientific: Quantum Ultra, Vantage; Agilent: 6495) - targeted proteomics method development for zebrafish embryo digests - measuring small compounds in zebrafish embryo extracts and in environmental water samples **Molecular biology** - qPCR - Western-blot **Bioassays** - zebrafish embryo toxicity test (zFET) - LPS challenge assay in zebrafish embryos (innate immune function) - ELISA - GR-CALUX® *in vitro* test - spectrophotometric assays (cell viability, Bradford, glucose measurement in zebrafish embryos, mitochondrial ROS production and complex I activity) **FELASA[2] Category B certificate (LTK Module 1)**
IT skills	GraphPad Prism, Skyline, Xcalibur, Uniprot, NCBI database, BLAST
Languages	English (fluent), German (intermediate), Hungarian (mother tongue)
Memberships	Swiss Chemical Society (SCS), Swiss Group for Mass Spectrometry (SGMS)

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[2] Federation of European laboratory Animal Science Associations

Conferences

SETAC Europe 24th Annual Meeting, 11-15 May, 2014, Basel, Switzerland. **Hidasi, A O**; Groh, K J; Suter, M J-F; Schirmer, K: *Novel approaches to assess the effects of glucocorticoids in zebrafish embryos* (Talk)

33rd SGMS 2015, October 29-30, 2015, Beatenberg, Switzerland. **Hidasi, A O**; Groh, K J; Suter, M J-F; Schirmer, K: *Targeted proteomics to find biomarkers of glucocorticoid exposure in zebrafish (Danio rerio) embryos* (Poster)

SETAC Europe 25th Annual Meeting, May 3-7, 2015, Barcelona, Spain. **Hidasi, A O**; Groh, K J; Tufi, S; Lamoree, M H; Leonards, P E G; Schirmer, K; Suter M J F: *Synthetic glucocorticoids in the aquatic environment: effects on zebrafish (Danio rerio) embryos* (Poster)

2nd CHanalysis 2015, 10-11 April, 2015, Beatenberg, Switzerland**. Hidasi, A O**; Tufi, S; Groh, K J; Schirmer, K; Lamoree, M H; Leonards, P E G; Suter M J F: *Omics methods to find biomarkers of glucocorticoid exposure in zebrafish (Danio rerio) embryos* (Poster)

20th International Mass Spectrometry Conference, 24-29 August, 2014, Geneva, Switzerland. **Hidasi, A O**; Groh, K J; Schirmer, K; Suter, M J-F: *Targeted proteomics approach to develop a bioassay detecting environmental glucocorticoids with zebrafish embryos* (Poster)

31st SGMS 2013, October 31 – November 1, 2013, Beatenberg, Switzerland. **Hidasi, A O**; Groh, K J; Suter, M J-F; Schirmer, K: *Targeted proteomics for monitoring glucocorticoid-specific protein biomarkers in zebrafish embryos* (Poster)

Publications

Hidasi, A O; Groh, K J; Suter, M J-F; Schirmer, K: Clobetasol propionate causes immunosuppression in zebrafish (*Danio rerio*) at environmentally relevant concentrations. – *Under review*

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