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Common deregulated gene expression profiles and morphological changes in developing zebrafish larvae exposed to environmental-relevant high to low concentrations of glucocorticoids

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HIGHLIGHTS

- Identified 159 unique homologs deregulated commonly by glucocorticoids.
- Homologs associated with over 20 molecular functions and 3 signalling pathways.
- Nervous, hepatic, endothelial-vascular and myeloid cell systems affected in larvae.
- *cry2b*, *fbxo32, klhl38b* responded robustly to environmental concentrations.
- Findings useful to infer biological impacts of glucocorticoid exposure in fish.

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ABSTRACT

Synthetic glucocorticoids have been detected in environmental waters and their biological potency have raised concerns of their impact on aquatic vertebrates especially fish. In this study, developing zebrafish larvae exposed to representative glucocorticoids (dexamethasone, prednisolone and triamcinolone) at 50 pM to 50 nM from 3 h post-fertilisation to 5 days post-fertilisation were investigated. Microarray analysis identified 1255, 1531, and 2380 gene probes, which correspondingly mapped to 660, 882 and 1238 human/rodent homologs, as deregulated by dexamethasone, prednisolone and triamcinolone, respectively. A total of 248 gene probes which mapped to 159 human/rodent homologs were commonly deregulated by the three glucocorticoids. These homologs were associated with over 20 molecular functions from cell cycle to cellular metabolisms, and were involved in the development and function of connective tissue, nervous, haematological, and digestive systems. Glucocorticoid receptor signalling, NRF2-mediated oxidative stress response and RAR signalling were among the top perturbed canonical pathways. Morphological analyses using four transgenic zebrafish lines revealed that the hepatic and endothelial-vascular systems were affected by all three glucocorticoids while nervous, pancreatic and myeloid cell systems were affected by one of them. Quantitative real-time PCR detected significant change in the expression of seven genes at 50 pM of all three glucocorticoids, a concentration comparable to total glucocorticoids reported in environmental waters. Three genes (*cry2b*, *fbxo32,* and *klhl38b*) responded robustly to all glucocorticoid concentrations tested. The common deregulated genes with the associated biological processes and morphological changes can be used for biological inference of glucocorticoid exposure in fish for future studies.

1. Introduction

Glucocorticoids (GCs) are steroidal compounds that can exert broad impact on vertebrate biology. Natural GCs, such as cortisol and cortisone, are produced in adrenal gland at basal levels under

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normal conditions and in response to stressors. They play essential roles in stress response and thereafter restore homeostasis by regulating the immune system, metabolism and body fluid (Granner et al., 2015). Synthetic GCs, such as dexamethasone (DEX), prednisolone (PRE) and triamcinolone (TRI) have been developed with enhanced potency and pharmacological properties, are commonly prescribed for anti-inflammatory and immunosuppressive therapies (Whitehouse, 2011). Depending on the type and preparation of drug as well as the targeted therapy, daily prescribed dose of GCs could range from 100 μg to 500 mg (Kugathas et al., 2012). The wide use of GCs and their incomplete removal during water treatment have led to the contamination of GCs in environmental waters raising the concern of their toxicities to the aquatic vertebrates. Total glucocorticoids levels have been detected at >0.5–52 ng/L in receiving river waters and up to 390 ng/L at discharged sites in Beijing region, China (Chang et al., 2009). Glucocorticoids and their metabolites totaling about 47–96 ng/L have been reported in Swiss river waters (Ammann et al., 2014). Cortisol and flumethasone have been detected up to 2.67 and 1.43 ng/L, respectively, in Danube River, Budapest, Hungary (Tolgyesi et al., 2010) while hydrocortisone at 7 ng/L was detected in Colorado river, Arizona, USA (Tolgyesi et al., 2010). In France, pharmaceutical industrial waste effluent detected with dexamethasone and prednisone up to 23,000 ng/L and 300 ng/L, respectively, have been discharged into a river where its downstream water were sampled over multiple time-points and estimated to contain 1–2900 ng/L of dexamethasone and 50–1260 ng/L of 6 α-methylprednisolone (Creusot et al., 2014). Moreover, total glucocorticoid levels between 30 and 850 ng/L have been predicted in the best and worst case scenario, respectively, in River Thames, UK (Kugathas et al., 2012).

The continuous and direct contact of aquatic vertebrates with GC contaminants in environmental water is a valid concern especially for fish (Benato et al., 2014). Recently, a complete mass balance of GCs and *in vitro* glucocorticoid receptor (GR) activity has been accomplished, which demonstrated previously unreported GCs at very low concentrations were extremely potent in a mammalian reporter-gene assay (Jia et al., 2016). Being a vertebrate, fish shares common developmental plan, organ systems including endocrine system and biological processes similar to mammals; hence fish are very likely to be sensitive to GCs. Consequently, there is an increasing attention on GC toxicity in fish. Although there have been many GC studies done at the molecular and physiological levels, mostly to understand their roles in health and diseases as well as their therapeutic effects in mammalian models (Harris, 2015), much less is known in fish. In mammals, overdose or prolonged GC exposure can cause severe side effects such as immunosuppression, hyperglycemia, muscular atrophy and developmental abnormalities (Schleimer, 1993; Franchimont, 2004; Korgun et al., 2012). In fish, adult fathead minnows exposed to two synthetic GCs for 21 days had elevated blood glucose and reduced leucocyte count significantly from 1.0 μg/L (~1.92 nM) onwards (Kugathas and Sumpter, 2011). Further study showed that female adult fathead minnows developed secondary male sexual characteristics and had reduced plasma vitellogenin concentration after exposure to as low as 0.1 μg/L (~192 pM) of beclomethasone dipropionate (BCMD) for 21 days (Kugathas et al., 2013). These studies have provided crucial physiological evidence of the impact in fish exposed chronically to synthetic GCs.

The effects of GCs and GR signalling have been investigated at the molecular level in zebrafish. The zebrafish (*Danio rerio*) is a popular model in developmental biology (Fishman, 2001; Lawrence, 2007), disease study (Ablain and Zon, 2013), and environmental toxicology (Stegeman et al., 2010). The ease of genetic manipulation, amenability to various molecular techniques and transgenic technology coupled with vast genomic resources have made zebrafish a model for research (Schaaf et al., 2009). Two GR isoforms with different regulatory functions have been identified in zebrafish (Schaaf et al., 2008; Chatzopoulou et al., 2015) and GC responsiveness in zebrafish have been demonstrated by GC-induced gene expression as well as in GC-responsive transgenic lines. Transgenic zebrafish lines responsive to GC have been developed using a promoter constructed with multiple GC-responsive elements fused with a fluorescent reporter gene (Weger et al., 2012; Benato et al., 2014; Krug et al., 2014). These transgenic lines have been developed for *in vivo* monitoring of endogenous GC activity or screening of GC drug candidates at high pharmacological doses (micromolar range). However, the strong background signal due to endogenous GC limits their sensitivity in detecting low concentration of GC or weak GC activity in the environment. GC responsiveness through induction of gene expression by exogenous GC at pharmacological doses of micromolar or high nanomolar range (Tseng et al., 2005; Elo et al., 2007; Mathew et al., 2007; Hillegass et al., 2008) to environmentally relevant concentrations of low nanomolar and picomolar range (Chen et al., 2016) have been demonstrated *in vivo* in zebrafish.

Instead of investigating GC responsiveness at single gene expression level, we are interested in studying GC responsiveness at the transcriptome level that will enable us to infer changes in biological processes as well as potential impact on physiological function and system in the zebrafish. To date, the published GC related transcriptomic studies in zebrafish did not focus on GC toxicity but have investigated mainly on the role of GR signalling during zebrafish embryonic development through GR knockdown in early embryos (Nesan and Vijayan, 2013) or via GR isoforms knockdown in zebrafish embryos treated with/without high pharmacological dose of 100 μM dexamethasone (Schaaf et al., 2008; Chatzopoulou et al., 2015). We, therefore, extended our investigation to GC-induced transcriptomic and morphological changes in zebrafish.

This study aimed to combine microarray-based transcriptomic and morphological analyses to investigate the effects of representative synthetic GCs on zebrafish embryos/larva exposed to nanomolar concentrations (50 nM and 5 nM) of DEX, PRE and TRI and eventually to test selected gene expression at picomolar concentrations. The nanomolar concentrations are comparable to the high end levels of total GC as estimated in some environmental waters (Kugathas et al., 2012; Creusot et al., 2014) although the usual levels are at picomolar range (Chang et al., 2009; Anumol et al., 2013; Ammann et al., 2014; Creusot et al., 2014; Jia et al., 2015). Given that GCs with different pharmacological properties have been detected in environmental water (Anumol et al., 2013; Creusot et al., 2014; Jia et al., 2016), this study also aimed to identify a common molecular signature shared by DEX, PRE and TRI as representative GCs. Knowledge-based functional analysis of individual and the common deregulated gene sets of the representative GCs were compared to infer common perturbed signalling pathways, biological functions and physiological systems. Relevant transgenic zebrafish lines with fluorescent tissue-organs exposed to the three representative GCs corroborated partly with the transcriptomic inference of perturbed biological functions and physiological systems. The expression of selected genes validated by real-time PCR corroborated with the perturbed signalling pathways. The expression of common deregulated genes that were further validated to be robust and sensitive even at environmental-relevant picomolar concentrations could serve as potential biomarkers of effects for GCs.

2. Materials and methods

2.1. Chemicals and reagents

Dexamethasone (DEX), prednisolone (PRE), and triamcinolone