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DIEL PATTERNS AND TISSUE-SPECIFICITY OF ENVIRONMENTAL RESPONSES IN FISH

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“An inspiration is a happy moment that takes us by surprise.”
Agnes Martin, artist
(22.3.1912–16.12.2004)

Contents

Abstract

Humans are profoundly changing aquatic environments through climate change and the release of nutrients and chemicals. To understand the effects of these changes on natural populations, knowledge on individuals' environmental responses is needed. At the molecular level, the environmental responses are partly mediated by chances in messenger RNA and protein levels. In this thesis I study messenger RNA and protein responses to an assortment of environmental stressors in fish. As daily (diel) rhythms are known to be ubiquitous in different tissues, I particularly focus on diel patterns in the responses. The studied species are the three-spined stickleback (*Gasterosteus aculeatus* L.) and the Arctic char (*Salvelinus alpinus* L.), both of which have circumpolar distribution in the Northern hemisphere.

In the first two studies, three-spined sticklebacks were exposed to both the non-steroidal anti-inflammatory drug diclofenac and low-oxygen conditions (hypoxia), and their responses measured at separate time points in the liver and gills. The results show how the seemingly unrelated environmental stressors, hypoxia and anti-inflammatory drugs, can have harmful combined effects that differ from the effects of each stressor alone. Moreover, both stressors disturbed natural diel patterns in gene expression.

In the third study, I studied the responses of three-spined sticklebacks to two test chemicals: one used in hormonal medicine (17α-ethinyl-oestradiol) and one used as a plasticizer and solvent chemical (di-n-butyl phthalate). The results suggest that the phthalate can affect genes related to spermatogenesis in fish testes, while estrogen-mimicking compounds can lead to numerous disturbances in the endocrine system.

In the final study, the temperature-dependence of diel rhythms in messenger RNA levels were evaluated in the liver tissue of the Arctic char, a cold-adapted salmonid. The results show that cold acclimation repressed diel rhythms in gene expression compared to warm-acclimated fish, in which the expression of hundreds of genes was rhythmic, suggesting the circadian clock of the Arctic fish species can be sensitive to temperature. Overall, the results of the thesis indicate that fishes' responses to abiotic factors interact with their diel rhythms, and more studies on the consequences of these interactions are needed to comprehensively understand human impacts on ecosystems.

Tiivistelmä

Ihmistoiminnan tuottamat kasvihuonekaasut sekä kemikaali- ja ravinnepäästöt muuttavat ympäristöä voimakkaasti. Muutosten vaikutusten ymmärtämiseksi tarvitsemme tietoa eri lajien ympäristövasteista. Molekyylitasolla vasteita säätelevät proteiinit, joiden määrä soluissa on osin riippuvainen tuotetun lähetti-RNA:n määrästä. Tässä väitöskirjassa käsittelen eri ympäristömuutosten vaikutuksia kaloihin lähetti-RNA:n määrän ja entsyymiaktiivisuuden tasolla eri kudoksissa. Vuorokausirytmien tiedetään säätelevän monien geenien ilmenemistä, joten keskityin etenkin ajalliseen vaihteluun kalojen ympäristövasteissa. Tutkitut kalalajit olivat lauhkeaan ilmastoon sopeutunut kolmipiikki (*Gasterosteus aculeatus* L.) ja arktisiin olosuhteisiin sopeutunut nieriä (*Salvelinus alpinus* L.). Kummatkin lajit elävät laajoilla alueilla pohjoisella pallonpuoliskolla.

Kahdessa ensimmäisessä työssä testasin kolmipiikkien vasteita jätevesissä esiintyvälle tulehduskipulääke diklofenaakille ja vähähappisille olosuhteille (hypoksialle). Maksa- ja kiduskudoksista saadut tulokset osoittavat hypoksialla ja diklofenaakilla olevan mahdollisesti haitallisia yhteisvaikutuksia kaloihin, ja että kiduksissa ja maksassa tapahtuvat muutokset voivat olla erisuuntaisia. Sekä diklofenaakin että hypoksian havaittiin myös muuttavan entsyymien luontaista päivärytmiä.

Kolmannessa osatyössä tutkin kolmipiikkien vasteita kahdelle hormonitoimintaa häiritseville yhdisteelle, keinotekoiselle estradiolille sekä muoviyhdisteissä käytetylle dibutyyliftalaatille. Havaitsin ftalaatin vaikuttavan siittiöiden toimintaa säätelevien geenien luentaan ja estradiolin estävän useiden steroidihormonien tuotantoa säätelevien geenien ilmenemistä kalojen sukuelimissä.

Viimeisessä osatyössä tutkin lämpenemisen vaikutuksia geeniluennan päivärytmiin lohikaloihin kuuluvalla nieriällä. Tulokset osoittivat, että korkeassa lämpötilassa nieriän geenien luenta vaihteli voimakkaasti päivän aikana, mutta viileässä lämpötilassa vaihtelu oli huomaamatonta, mikä viittaa siihen, että arktisten kalojen biologinen kello voi olla herkkä lämpötilamuutoksille. Kokonaisuudessaan väitöskirjan tulokset osoittavat, että meidän on vaikea ennustaa ympäristömuutosten yhteisvaikutuksia luonnon populaatioihin, jos emme ymmärrä ympäristövasteiden ja biologisten rytmien vuorovaikutuksia.

List of original articles and author contributions

I. **Prokkola JM**, Nikinmaa M, Lubiana P, Kanerva M, McCairns RJS, Götting M (2015) Hypoxia and the pharmaceutical diclofenac influence the circadian responses of three-spined stickleback. *Aquatic Toxicology* 158:116–124. DOI: 10.1016/j.aquatox.2014.11.006.

II. Lubiana P*, **Prokkola JM***, Nikinmaa M, Burmester T, Kanerva M, Götting M. The effects of the painkiller diclofenac and hypoxia on gene transcription and antioxidant system in the gills of three-spined stickleback. Manuscript.

III. **Prokkola JM**, Katsiadaki I, Sebire M, Elphinstone-Davis J, Pausio S, Nikinmaa M, Leder EH (2016) Microarray analysis of di-n-butyl phthalate and 17 α ethinyl-oestradiol responses in three-spined stickleback testes reveals novel candidate genes for endocrine disruption. *Ecotoxicology and Environmental Safety* 124: 96–104. DOI: 10.1016/j.ecoenv.2015.09.039.

IV. **Prokkola JM**, Nikinmaa M, Lewis M, Anttila K, Kanerva M, Ikkala K, Seppänen E, Kolari I, Leder EH. Cold temperature represses diel rhythms in Arctic char (*Salvelinus alpinus*) transcriptome despite light-dark rhythm. Manuscript.

* Equal contribution to the article.
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Contributions to the original articles.

	I	II	III	IV
Study design	MG, JP, PL, MN	MG, JP, PL, MN	IK, MS	JP, MN, KA, ML
Experiment	MG, PL, JP	MG, PL, JP	IK, MS, JED, JP	ML, KA, JP, MK, KI, IK, ES
Laboratory	JP, MK	PL, MG, MK	SP	JP
Data analysis	JP, RJSM	JP, MG	JP, EHL, SP	JP, EHL
Manuscript	JP, MN, PL, MG, RJSM	PL, MG, JP, MN, TB, MK	JP, EHL, MN, IK	JP, EHL, MN, KA, ES, KI, ML

Abbreviations

AHR	Aryl hydrocarbon receptor
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
CAT	Catalase
CRY	Cryptochrome
CYP1A	Cytochrome P450 1A
DBP	Di-n-butyl phthalate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EE2	17-alpha ethinyl-oestradiol
ENO	Enolase
EROD	Ethoxyresorufin-O-deethylase
GPX	Glutathione peroxidase
HIF	Hypoxia-inducible factor
HSP	Heat shock protein
LDH	Lactate dehydrogenase
ORF	Open reading frame
PAS	PER-ARNT-SIM domain
PE	Paired-end
PER	Period
RIN	RNA integrity number
RNA	Ribonucleic acid
ROS	Reactive oxygen species
qPCR	Quantitative real-time polymerase chain reaction
SOD	Superoxide dismutase

1. Introduction

Organisms are constantly interacting with their environment. As the environment changes, populations respond by adapting to the novel conditions, altering their habitat range, migrating, increasing or decreasing in size or by going locally extinct. Population-scale responses are the outcome of physiological responses of individuals. Direct genetic effects and plasticity in the phenotype, which is partially regulated by epigenetic mechanisms, influence individual responses and fitness. Fitness differences among individuals in a population attributed to genetic variation result in evolutionary changes in genotype frequencies over generations. Thus, historical environmental conditions have shaped the tolerance of extant species to variation in abiotic environmental conditions (Bijlsma and Loeschcke 2005), such as temperature, oxygen availability and pH. Since the global changes create novel environments different to those experienced by extant species during their evolutionary history (Hobbs et al. 2006, Williams et al. 2007), it is now more important than ever to understand the physiological mechanisms by which organisms respond to various stressors and their limits.

Since the industrial revolution, human impact on the environment has increased rapidly, to the extent that the on-going era, characterized by human domination on Earth, has been informally named as the Anthropocene (Zalasiewicz et al. 2010). Recent climate change, caused largely by the increasing release of carbon dioxide and other greenhouse gases to the atmosphere, is accelerating and has effects on ecosystems, sea level, agriculture and human livelihood around the planet (IPCC, 2014). Increased human and domestic animal populations and decreased carbon dioxide consumption by autotrophic organisms both contribute to the change, which encompasses global warming and several other climatic phenomena and the associated ocean acidification (Harley et al. 2006, Trenberth 2011). The negatively affected species are predicted to be those whose habitat is currently limited by temperature or other abiotic and biotic factors now being altered by climate change (Hannah et al. 2007, Rahel et al. 2008), but also those in which evolutionary stasis prevents adaptation of key traits necessary for survival (Reusch and Wood 2007). Generally, the most threatening combination for populations arises from habitat fragmentation combined with the rapid climate change (Travis 2003).

Global warming is projected to increase the surface temperature of the Earth by approximately 1.5–4 °C during the 21st century, with the most rapid increase occurring in the Arctic (IPCC, 2014). Warming is driving changes in the spatial distribution or phenology of species by changing habitat characteristics (Parmesan and Yohe 2003). In aquatic environments, a rise in temperature decreases oxygen availability, which further decreases survival in cold-adapted species already challenged by high temperatures. An additional decrease in oxygen availability can be induced by the release of large quantities of nutrients from agriculture and wastewater. In a warm environment, the nutrients are rapidly bound to biomass. The decomposition of large amounts of biomass consumes more oxygen than is provided by diffusion and photosynthesis, leading to environmental oxygen shortage (hypoxia) or a total lack of oxygen (anoxia).

In high latitudes, hypoxia caused by decomposing biomass is especially pronounced in the late summer, whereas in the winter ice cover prohibits oxygen from dissolving from the atmosphere, causing a sustained, long-term hypoxia or anoxia. In addition, circadian (from *circa* -about, *diem* -day) variation in oxygen availability during the growth season can be significant in eutrophic aquatic systems in temperate regions and the tropics (Nikinmaa and Rees 2005). During the day, when sunlight is available, photosynthesis by primary producers maintains normal oxygen levels (normoxia), or creates an excess of oxygen (hyperoxia). During the night, photosynthesis ceases, while all organisms in the ecosystems continue to consume oxygen, which rapidly leads to decreased oxygen levels. In addition, ecosystems in several marine coastal areas are at risk due to a more permanent hypoxia, caused by a stratification of the water column (Helly and Levin 2004).

Coinciding with recent climate change, humans have introduced thousands of chemicals to the environment, a fraction of which have been studied for risks related to sub-lethal effects in the ecosystems. In developed countries, exposure to low levels of low-persistence chemicals, including pharmaceuticals and personal-care products, is considered as the least understood environmental risk by chemicals (Daughton and Ternes 1999). Many of these substances, together with plastics, are nearly ubiquitously present in aquatic environments around the world (Andrady 2011, Corcoran 2015).

In the environment, organisms are faced with multiple abiotic changes simultaneously, and their responses to the factors are not isolated. For example, hypoxia and warm acclimation have species-specific effects on the tolerance to each stressor in salmonids (Anttila et al. 2015), exposure to heat stress can protect fish from osmotic stress in tidal pools (Todgham et al. 2005) and the co-effects of hypoxia and pollutants can be additive, synergistic or inhibitory compared to exposures to single stressors (Matson et al. 2008, Celander 2011, Gauthier et al. 2014, Song et al. 2014, Sappal et al. 2015). Hence, the acclimation abilities of aquatic species are under pressure by the constant release of chemicals, increasing temperatures and frequent hypoxia. To understand why unrelated environmental stressors can have so-called cocktail effects on organisms, we must tease apart the factors that determine how genes and proteins interact at the lowest level of complexity (Fig. 1). This knowledge can help understand how entire populations and species are affected by the current environmental changes.

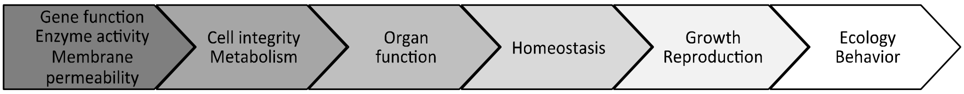

Figure 1. The levels of biological complexity from molecular to organismal level. Complexity increases from left to right, and often our understanding of how a multitude of simple responses generate the complex processes, such as disrupted reproduction by pollutants, is limited. Modified from Heath (1995).

1.1. Genomic regulation of protein function

Environmental responses of organisms are mediated by changes in protein activity and abundance. Proteins make up most of the structures of organisms and carry out all the biological functions by acting as, e.g., hormones, transcription factors and receptors that respond to changes in environmental conditions to adjust behavior and the synthesis and degradation of molecules in tissues. The deoxyribonucleic acid (DNA) sequence mainly determines the amino acid sequence of a protein, but specific enzymes can change individual amino acids in the protein sequence by editing the mRNA before translation (Garrett and Rosenthal, 2012). In the protein the elements that define its function are called domains. For environmental responses, a domain called Period-Arnt-Sim (PAS) is particularly important, since the key transcription factors involved in circadian rhythms and the cellular responses to hypoxia and chemical detoxification all contain PAS domains (McIntosh et al. 2010). PAS domains allow these proteins to recognize each other and facilitate the formation of specific protein homo- and heterodimers, which is a necessary step in target gene activation for many environmentally relevant PAS proteins (Möglich et al. 2009).

Proteins are produced in a multi-step process called gene expression (Fig. 2). In the first step, messenger ribonucleic acid (mRNA) is transcribed from the DNA template by RNA polymerase enzymes. Next, ribosomes combine single amino acids carried by transfer-RNAs into a polypeptide during translation. The abundance of mRNAs and the rate of translation are regarded as the most important determinants of the abundance of a protein in cells, and recent studies have shown mRNA level to explain approximately 40% – 86% of variation in protein abundance (Schwanhaeusser et al. 2011, Li et al. 2014, Csardi et al. 2015). The rate of translation can be attenuated globally through the phosphorylation of eukaryotic initiation factor 2α, while the availability of mRNA for translation is regulated by multiple global and gene-specific mechanisms (Sonenberg and Hinnebusch, 2007). Notably, the relationship between mRNA and protein is also species- and protein-specific; for example different species of salmonids respond to heat stress by increasing both the mRNA and protein abundance or only the mRNA abundance of heat shock proteins (Mario Lewis, personal communication).

Different non-protein-coding RNAs affect the availability of mRNA for translation. These include micro-RNAs, which are on average only 22 base pairs (bp) in length, and the so-called long non-coding RNAs (lnc-RNAs), which can be hundreds of bp long. Micro-RNAs silence the expression of target genes by affecting mRNA stability and inhibiting the translation of mRNA to protein (Valencia-Sanchez et al. 2006, Guo et al. 2010), while lnc-RNAs can facilitate epigenetic modifications of DNA and premature termination of transcription or modify protein activity directly (Mercer et al. 2009, Kornfeld and Bruening 2014). Alternative folding of the translated peptides and the degradation of proteins is used as another level of regulation of gene expression. The process of generating a functional protein product based on DNA sequence is called gene expression, although the term is frequently used in reference to the expression of mRNA.

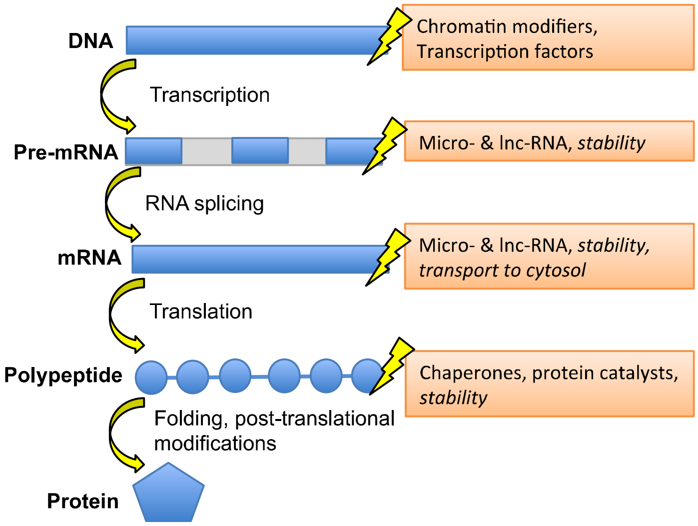

Figure 2. Illustration of the stages in gene expression from DNA to the final protein product. The quantity of each intermediate product is affected by the rate of the preceding process as well as the factors depicted in the orange boxes.

In environmental physiology, the abundance of proteins or the intermediate products of gene expression, such as mRNA, are often measured to gain an insight into the molecular regulators of environmental responses and their differentiation among populations (Schulte 2004, Larsen et al. 2011, Rees et al. 2011). The methods for quantifying protein and RNA abundances have advanced tremendously in the past few decades due to the introduction of mass-spectrometry-based methods for protein quantification, and microarray and Next Generation Sequencing (NGS) -based methods for quantifying different types of RNA (Bowtell 1999, Aebersold and Mann 2003, Wang et al. 2009). With microarray and RNA-sequencing technologies it has become possible to quantify mRNAs at the whole genome level. At the individual gene level, quantitative real-time polymerase chain reaction (qPCR) is widely used to quantify the abundance of individual mRNA sequences (Bustin et al. 2005), and the costs of this method are a fraction of the genome-wide technologies. In contrast to measuring RNA abundance, measuring protein activity, the rate at which enzymes perform, can only be performed using “low-throughput” methodology, i.e., by measuring each enzyme activity individually from a tissue homogenate. Only by this method can protein performance, i.e., its function, be estimated (Nikinmaa and Rytkönen 2012). Combining both low-throughput (e.g, fluorescence spectrophotometry and qPCR) and high-throughput (e.g, NGS- and microarray-based) methods can be regarded as a comprehensive method for studying the dynamics involved in the process of gene expression.

Despite the rapid advances taken at understanding molecular environmental responses between and within populations, temporal variability in gene expression introduced by endogenous time-keeping mechanisms is still poorly known. Disturbances in adaptive, temporal gene expression patterns may introduce a new level of costs that are not considered in many environmental physiology and toxicology studies. In addition, overlooking this variation can have implications for the repeatability and generality of observations made under constant conditions.

1.2. Circadian rhythms in vertebrates

Circadian rhythms are 24-h cycles in physiology and behavior followed by nearly all species on Earth –across all domains from *Eukarya* and *Bacteria* to *Archaea* (Lowrey and Takahashi 2004, Whitehead et al 2009). Circadian rhythms are expressed in one of the oldest life forms on our planet, the cyanobacteria, suggesting an ancient origin for biological rhythms (Ditty et al. 2003). Within the animal kingdom, circadian rhythms originate from a time before the previous common ancestor of mammals and insects, as shown by the similarities in the circadian systems between these lineages (Rosbash 2009).

The advantage that has allowed circadian rhythms to spread so widely in different species is that they allow organisms to anticipate rhythmic changes in their environments, such as temperature and light, thus optimizing their energy use by the timing of metabolism and behavior. For example, cell cycle, DNA repair, immunity and the secretion of several hormones are in part controlled by circadian rhythms (Plytycz and Seljelid 1997, Falcon et al. 2011, Challet 2015). Many rhythmic processes are regulated by the hormone melatonin, which in many vertebrate taxa is secreted from the pineal gland

and regulated by the central circadian clock *via* noradrenergic signaling (Simonneaux and Ribelayga 2003, Falcon et al. 2011).

The central clock, which is located in the suprachiasmatic nucleus of the hypothalamus in mammals, and in the pineal gland in fish, acts as a master regulator of the rhythms in other tissues, referred to as the peripheral rhythms or "slave oscillators" (Reppert and Weaver 2002, Idda et al. 2012). The central clock responds to environmental zeitgebers (time-givers), the most potent of which is the light dark rhythm (Reppert and Weaver 2002). To allow for a tissue-specific, coordinated timing of different metabolic and catabolic reactions, different tissues obey their own endogenous circadian rhythms (Lowrey and Takahashi 2004).

The transcriptional engine of the circadian clock is similar in the central and peripheral clocks, and highly conserved in vertebrates. It can be simplified into two feedback loops (Fig. 3): a positive loop, involving the proteins CLOCK and cycle (CYC, previously brain-and-muscle-arntl-like, BMAL), and a negative loop, including the proteins cryptochrome (CRY) and period (PER), all of which are present in two or more different gene copies in vertebrates (Lowrey and Takahashi 2004, McIntosh et al. 2010). In the positive loop, the constitutively expressed CLOCK and CYC dimerize and bind to E-box elements in DNA, thereby regulating the expression of a variety of genes, including *Per* and *Cry*. In the negative loop, the PER and CRY proteins dimerize, and as they increase in abundance, inhibit the activation of the CLOCK-CYC-complex, and consequently their own expression, which ultimately allows the positive loop to reinitiate the rhythm (Lowrey and Takahashi 2004, McIntosh et al. 2010).

Circadian oscillators in teleost fish are largely homologous to those in mammals, but the number of genes in each component varies across species. The genome of teleost fish went through a whole-genome duplication event approximately 330-400 Million years ago (Mya) (Hoegg et al. 2004), after which the lineages of at least salmonids and cyprinids have experienced additional genome duplications, approximately 100 and 10 Mya, respectively (Danzmann et al. 2008, Ma et al. 2014, Macqueen and Johnston 2014). An analysis on the diversification rate of the clock gene paralogs has found that most have gone through significant diversification or have been under relaxed purifying selection since the genome duplications, suggesting the encoded proteins may have diversified roles in circadian clock regulation (Wang 2008a, b, 2009). However, the precise roles of circadian clock gene paralogs in teleost fish are relatively well understood only in zebrafish (Vatine et al. 2011). Scattered evidence exists regarding the clock components' expression in other species, including goldfish (*Carassius auratus*), Atlantic salmon (*Salmo salar*) and European seabass (*Dicentrarchus labrax*) (Velarde et al. 2009, Huang et al. 2010, Feliciano et al. 2011, McStay et al. 2014). A few genetic studies have identified the *clock* gene as a putative regulator of spawning time and migration, as well as having different alleles across a latitudinal gradient in salmonids (Leder et al. 2006, O'Malley and Banks 2008, O'Malley et al. 2014). Thus, circadian clock genes appear to be essential for the timing of not only circadian rhythms, but also seasonality in fish.

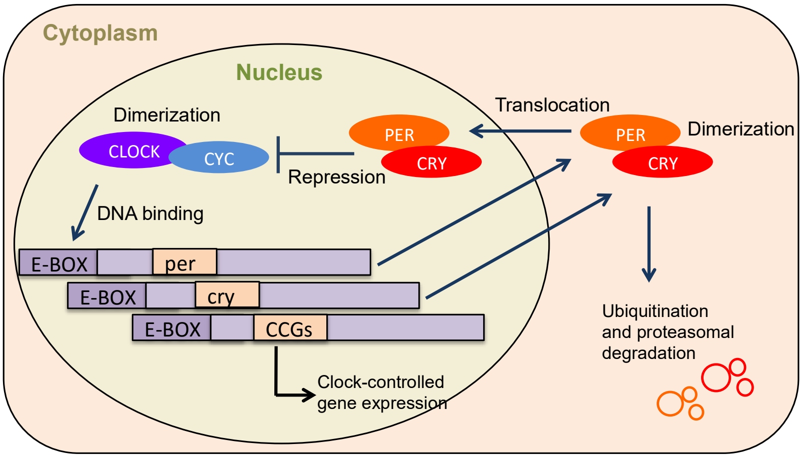

Figure 3. Schematic illustration of the feedback loops regulating circadian gene expression in vertebrates. Constitutively expressed CLOCK dimerizes with CYC in the nucleus, and binds to E-box elements in the promoter regions of *per*, *cry* and clock-controlled genes (CGGs). PER and CRY dimerize in the cytoplasm and the dimer translocates to the nucleus. The accumulation of PER-CRY dimer is partly controlled by ubiquitination and degradation of the proteins. In the nucleus, PER and CRY inhibit the activity of CLOCK-CYC dimer, eventually allowing the transcriptional cycle to restart. CYC expression is also modified by a third transcriptional loop including the proteins ROR-α and REV-ERBα (not shown). Modified from Mohawk et al. (2012).

1.3. Molecular responses to hypoxia

The aerobic mode of glucose metabolism is preferred to anaerobic metabolism by most organisms, since the adenosine triphosphate (ATP) yield of anaerobic respiration is only about 6% of the ATP produced aerobically. Nevertheless, when oxygen is not available to meet the needs of aerobic ATP production, several mechanisms have evolved to facilitate survival in short- and long-term hypoxic challenges. Besides avoiding environments where oxygen demand is not met, reducing oxygen consumption is one of the most important strategies for survival during hypoxia and anoxia. It can be observed in fish as reduced activity and feeding and, in the long-term, disrupted growth (Breitburg 2002).

From mammals to fish, one transcription factor has been described as a master regulator of hypoxia acclimation: the hypoxia-inducible factor 1 (HIF-1) (Semenza and Wang 1992, Soitamo et al. 2001, Semenza 2004). The regulation of HIF-1 is a well-known process. The subunits of HIF-1 are named

HIF-1α and aryl hydrocarbon nuclear translocator (ARNT, previously known as HIF-1β) (Fig. 4).

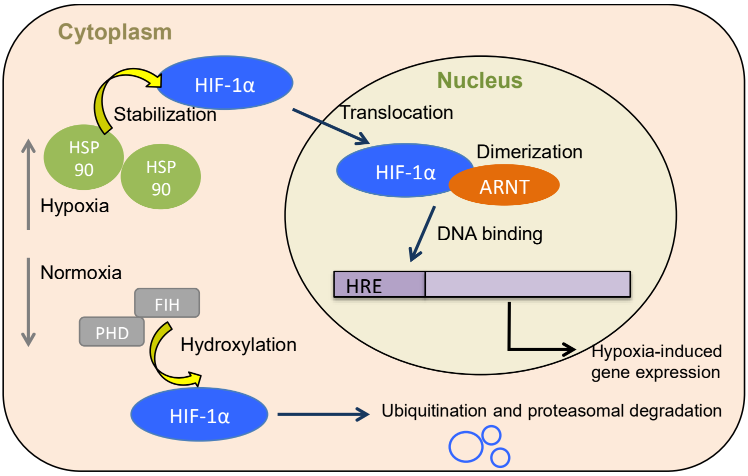

Figure 4. Schematic presentation of HIF-1a regulation under hypoxic and normoxic conditions. In normoxia, constitutively expressed HIF-1α is hydroxylated by factor-inhibiting-HIF (FIH) and proline hydroxylase 2 (PHD), followed by ubiquitination and degradation by proteasomal complex. In hypoxia, HIF-1α is stabilized by a process including heat shock protein 90 (HSP90) (although the precise mechanism is not well known) and transported to the nucleus, where it dimerizes with ARNT, forming the active HIF-1 complex. HIF-1 binds to DNA in hypoxia-response elements (HREs) upstream from the coding regions of target genes and activates gene expression. Modified from McIntosh et al. (2010).

The main HIF-1 regulatory step is the oxygen-dependent accumulation of HIF-1α protein, which is regulated mainly at the post-transcriptional level in mammals, likely as an adaptation to maximize the efficiency of a hypoxia response, when spending time on protein translation and mRNA transcription could be detrimental to the organism. The regulation of target gene transcription by HIF-1 follows a common pattern of PAS-proteins, which often act as heterodimers with other members of the PAS-protein family. The recognition of the target genes occurs through motifs called hypoxia-response elements (Nikinmaa and Rees 2005). It has been estimated that over 200 genes in the human genome have a varying number of these elements that regulate their expression, which is compatible with the estimates of the number of hypoxia-regulated genes (Chowdhury et al. 2008, Ortiz-Barahona et al. 2010). Among the most thoroughly studied HIF-1 target genes are erythropoietin, vascular endothelial growth factor and lactate dehydrogenase, which generate acclimation to hypoxia at the systemic, tissue and cellular levels (Semenza 2004, Nikinmaa and Rees 2005). These genes encode proteins that increase the number of red blood cells in circulation, the growth of blood vessels and the conversion of pyruvate to lactate in anaerobic metabolism, respectively. However, the list of HIF-1 target genes is expanding, and more targets are being identified using high-throughput methods and studying responses in different species, life-stages and tissues.

Recently, molecular interactions have been described between HIF-1 and one of the circadian clock components, PER1 in zebrafish (Egg et al. 2013). Both *hif-1α* mRNA and protein accumulation were shown to depend on the timing of hypoxia exposure in zebrafish, while HIF-1 was also shown to regulate oscillation in *per1* mRNA (Egg et al. 2013, Pelster and Egg 2015). These results suggest firstly that HIF-1 accumulation can be under circadian regulation, and secondly that exposure to hypoxia can disrupt the amplitude or rhythm of circadian clock gene expression. Yet, little is known of the natural circadian variation in enzymes and proteins associated to hypoxia response in fish adapted to seasonal habitats.

1.4. Reactive oxygen species in environmental responses

Reactive oxygen species (ROS), including hydrogen peroxide H_2O_2, superoxide anion $O_2^{\bullet-}$ and others, have recently emerged as a set of cellular signaling molecules. They can be produced in several enzyme reactions, and are also released in small amounts from the electron transport chain in mitochondria during aerobic respiration (Valko et al. 2007). As a signal of the metabolic state of cell, ROS level is used in the integration of metabolism and other cellular processes, such as circadian rhythms (Rutter et al. 2002, Edgar et al. 2012) and immunity (Zhou et al. 2015). When produced in excess, ROS cause oxidative stress, which can have negative effects on the integrity of cell membranes and proteins (Valko et al. 2007). Thus, ROS are both essential and harmful to cellular homeostasis (Dröge 2002). Cells are equipped to counter-balance the production of ROS to prevent oxidative damage and maintain redox homeostasis by using enzymatic and non-enzymatic antioxidants, which catalyze the conversion of ROS to molecular oxygen O_2 via different pathways.

The main antioxidant enzymes are highly conserved across taxa, and include superoxide dismutase (SOD), catalase (CAT) and several enzymes that maintain the redox status of glutathione, such as glutathione peroxidase (GPx). Glutathione is a tripeptide that is used as the main antioxidant in the cytosol and mitochondria and as a cofactor of the GpX enzyme (Valko et al. 2007). SOD enzymes convert the superoxide anion to hydrogen peroxide (Deby and Goutier 1990), which is thereafter converted to water by CAT enzymes and GPx, which is more sensitive to H_2O_2 than CAT (Deby and Goutier 1990, Mates 2000, Limon-Pacheco and Gonsebatt 2009). The activity of antioxidant enzymes can be measured by spectrophotometry, and an increase in their activity usually indicates an increase in ROS levels, which is a signal of oxidative stress (Lushchak 2011).

The association of ROS and hypoxia has been under intense research since the mid-1990s. ROS has been shown to increase during the re-oxygenation period after hypoxia exposure (Bickler and Buck 2007). Since some fish were found to increase antioxidant enzyme activity during hypoxia, this was interpreted as an anticipatory response to re-oxygenation, as hypoxia is generally expected to decrease ROS. However, this response was found to be largely tissue-specific (Lushchak et al. 2005, Lushchak and Bagnyukova 2006, Lushchak 2011). Recently, it was proposed that ROS formation induces antioxidant enzyme activities at a specific oxygen level during the early stages of hypoxia, prior to anoxia and similarly during re-oxygenation, and that the critical oxygen level is species and tissue-dependent (Hermes-Lima et al. 2015). Variation in the critical oxygen level at which ROS formation is increased could explain why no consistent changes in antioxidant activities were found in many studies on moderately to highly hypoxia tolerant species, e.g., (Lushchak et al. 2005, Lushchak and Bagnyukova 2006, Leveelahti et al. 2014, Hermes-Lima et al. 2015).

During hypoxia, antioxidants can help maintain a reducing environment in the cytoplasm, which is important for the stability of several transcription factors, including HIF-1α (Nikinmaa et al. 2004, Trachootham et al. 2008). Pollutants can also lead to increased ROS and have effects on cellular reduced/oxidized (redox) status (Lushchak 2011), which can in turn affect the responses of organisms to hypoxia (Nikinmaa et al. 2004). Furthermore, cellular redox status has been linked to circadian rhythm signaling; they do share their evolutionary history ever since the Great Oxygenation Event, approximately 2.5 billion years ago (Milev and Reddy 2015). Circadian rhythms in metabolism generate oscillations in the cellular redox status, which can be sensed and adjusted by core circadian clock genes (Milev and Reddy 2015). Even though circadian rhythms have been described for some antioxidants in rodents (Belanger et al. 1991, NeuschwanderTetri and Rozin 1996), we know very little of the relationship between antioxidant enzymes and the circadian oscillation in ROS during normoxic and hypoxic conditions in other species. Nevertheless, due to the numerous connections between circadian rhythms, redox status and environmental responses, it is clear that a delicate control in generation and elimination of ROS is essential for organisms.

1.5. Micropollutants in aquatic environments and their effects on fish

Aquatic ecosystems collect traces of all chemicals that can be dissolved in water or bound to particles carried in effluent water, agricultural run-off or rainfall. Fish are susceptible to pollutants, as dissolved compounds can enter their circulation through gills, skin or ingested food. The detoxification mechanisms in different tissues of fish are efficient in removing lipophilic compounds, and different enzymatic and non-enzymatic repair mechanisms and antioxidants aim to minimize adverse effects on tissue function and homeostasis. Despite these efforts, negative effects on tissue function, reproduction and behavior, as well as increased cancer incidence have been reported in wild populations of fish in polluted areas (Vos et al. 2000, Vethaak et al. 2009, Floehr et al. 2015). Furthermore, aquatic pollution can be a driver of natural selection in the wild; long-term effects of pollution have been associated with falling trout population sizes in Lake Ontario (Cook et al. 2003), and populations of killifish (*Fundulus heteroclitus*) show adaptation to polluted environments in their DNA sequence and gene expression levels (Williams and Oleksiak 2008, Whitehead et al. 2010).

Micropollutants is a term that encompasses a wide range of emerging pollutants that are found in the environment in low, nanogram to microgram per L concentrations (Luo et al. 2014). These are usually compounds that are used as pharmaceuticals, plasticizers, insecticides or personal care products. Pharmaceuticals are transmitted into the environment through inappropriate disposal and urine, as our bodies generally metabolize roughly only 50% of pharmaceuticals before excretion (Lienert et al. 2007). Where available, wastewater treatment plants remove varying amounts of pharmaceuticals from the incoming water, and flooding events and heavy rainfall can further weaken the removal (Daughton and Ternes 1999). Consequently, a high number of pharmaceutical substances have been detected in both wastewater effluent and surface waters, (e.g., Tixier et al. (2003), Loos et al. (2013) and Kleywegt et al. (2011)). To make environmental risks worse, pharmaceuticals have been designed to target specific biological pathways in very low concentrations and be resistant to biological degradation —both of which are desired qualities for a therapeutic agent, but can increase the risk of harmful effects in non-target species (Daughton and Ternes 1999).

Non-steroidal anti-inflammatory drugs (NSAIDs) are some of the most frequently encountered pharmaceuticals in wastewater effluent. Their use for human veterinary care is increasingly common around the world. Among the most commonly consumed NSAIDs are ibuprofen, acetaminophen and diclofenac (Luo et al. 2014). Of these, diclofenac has raised the most concern regarding environmental effects, since it has been shown to accumulate in the bile of wild fish and have histopathological effects at low, 1−5 µg/L concentrations in rainbow trout and brown trout (*Salmo trutta fario*) (Schwaiger et al. 2004, Triebskorn et al. 2004, Hoeger et al. 2005, Brozinski et al. 2013). In 2013, The European Union (EU) selected diclofenac as one of the watch list substances to be potentially included in the water framework directive with the goal of preventing harmful effects in aquatic wildlife (Ribeiro et al. 2015). Diclofenac has been frequently measured in wastewater effluent at approximately 1-µg/L concentrations (Scheurell et al. 2009, Stasinakis et al. 2012, Luo et al. 2014).

Oxidative stress can contribute to the toxicity of NSAIDs in humans (Amacher 2006), and recently increased oxidative damage and activities of antioxidant enzymes after diclofenac exposure were described in the common carp (*Cyprinus carpio*) (Saucedo-Vence et al. 2015). Another study has shown effects on hepatic *cytochrome P450 1A* (*cyp1a*) and *cyclooxygenase* (*cox*, a.k.a *prostaglandin-endoperoxide synthase)*, transcription by diclofenac in rainbow trout (Mehinto et al. 2010). CYP1A is a mixed function oxidase enzyme that detoxifies dioxin- and polycyclic aromatic hydrocarbon-like compounds (Celander 2011). Its expression is transcriptionally regulated via the aryl hydrocarbon receptor (AHR), which is a member of the PAS-protein family (Schmidt and Bradfield 1996). *Cox1* and *cox2* are inhibited by most NSAIDs, leading to decreased prostaglandin synthesis, which decreases inflammation and pain.

A number of micropollutants can affect the hormonal system of non-target species acting as endocrine disrupters. For instance, xeno-estrogens can induce feminization in male fish through estrogen receptors (Laenge et al. 2001, Van den Belt et al. 2001, Rajapakse et al. 2002, Grist et al. 2003, Balch et al. 2004), while anti-androgens, including several plasticizers and solvents, can induce feminization by inhibiting androgen receptor activity and testosterone synthesis (Kelce and Wilson 1997, Shultz et al. 2001). One of the most widespread xeno-estrogens in the environment is 17α ethinyl-oestradiol (EE2), which has been widely used in hormonal contraceptives. In fish, the no-observed-effect concentrations (NOEC) for chronic EE2 exposure are as low as 1 ng/L (Länge et al. 2001, Grist et al. 2003). The most common physiological effects of EE2 include inhibition of testosterone-producing enzymes and decreased levels of testosterone (Martyniuk et al. 2006, Filby et al. 2007, Garcia-Reyero et al. 2009, Reyhanian et al. 2011, Doyle et al. 2013). EE2 and other natural and synthetic estrogens have repeatedly been measured in WWTP effluent around the world at several ng/L concentrations (Ternes et al. 1999, Martin et al. 2012, Eugenia Valdes et al. 2015).

Phthalate esters are widely used plasticizers and solvents in cosmetics and other consumer products (Berge et al. 2013). Severe endocrine effects by phthalates have been reported in rats (van den Driesche et al. 2012) and indication of anti-androgenicity by di-n-butyl phthalate (DBP) has been described in three-spined sticklebacks (Aoki et al. 2011). However, the mechanisms behind the anti-androgenic effects of DBP are not understood (Mankidy et al. 2013). Different phthalates, mostly di-2-ethylhexyl phthalate and DBP, have been frequently found in wastewater effluent, sludge, soil, sediments and surface waters, at up to several microgram per liter concentrations (Fromme et al. 2002, Roslev et al. 2007, Berge et al. 2013, Benjamin et al. 2015).

In addition to direct effects through detoxification and endocrine disruption, xenobiotics can have indirect effects on organisms through disturbances in circadian rhythms or responses to other stressors. Biomedical studies have revealed that circadian rhythms have major effects on drug metabolism in humans, and *vice versa*, that certain drugs can disturb circadian oscillators (Claudel et al. 2007). In mangrove killifish (*Kryptolebias marmoratus*), circadian clock gene mRNA levels were disrupted by exposure to endocrine disrupting chemicals (Rhee et al. 2014). Likewise, co-exposures to pollutants and hypoxia were shown to induce distinct effects on oxidative damage and antioxidant enzymes compared to single exposures in pacu (*Piaractus mesopotamicus*) and orange-spotted grouper (*Epinephelus coioides*) (Sampaio et al. 2008, Yu et al. 2008). No studies thus far have simultaneously addressed the potential interactions of hypoxia responses, chemical detoxification and the circadian clock in fish, which is a major gap in our understanding of dynamics behind environmental responses in aquatic species.

1.6. The interrelationship of temperature and light at high latitudes

At high latitudes, seasonal changes in temperature are coupled to variation in the light-dark cycle. In the polar regions, the light-dark cycle is undetectable for approximately one third of the year, but many species in these habitats have persistent circadian rhythms despite the lack of environmental cues (Williams et al. 2015). Others, instead, lose observable circadian rhythms in activity or circadian clock gene expression during the polar day (Lu et al. 2010, Kobelkova et al. 2015, Williams et al. 2015). During fall and spring, the light-dark rhythm acts as a cue for seasonal changes in ambient temperature, which affects for example food abundance and, for ectotherms, also metabolic rate (Clarke and Johnston 1999). Climate change is now creating a mismatch between the observed day-length and anticipated temperature, which poses a challenge for survival in species living in highly seasonal habitats (Stevenson et al. 2015). For species adapted to cold, the challenge is inflated with the negative effects related to facing temperatures at their upper temperature limits (Somero 2010, Stevenson et al. 2015).

Fish are characterized as eurythermal or stenothermal based on whether they tolerate large or narrow variations in temperature (Logan and Buckley 2015). Eurythermal fish respond to changes in ambient temperatures with a multitude of physiological responses ranging from cardiac output to transcriptional regulation (see, e.g., Lee et al. 2003, Franklin et al. 2007, Smith et al. 2013, Anttila et al. 2014 and Schulte 2015). In cold-adapted stenothermal fish from polar regions, temperature responses can be distinct from eurythermal fish (Logan and Buckley 2015). For instance, Antarctic fish express heat-shock proteins constitutively, possibly to cope with severe cold (Hofmann et al. 2000, Place et al. 2004). It is therefore urgent to understand how and which processes show variation in response to temperature and light rhythm in species adapted to seasonal environments. Particularly Arctic fish require research on this front, as little is known of the physiology behind their seasonality, yet the climate is warming especially rapidly in their habitat (Jørgensen and Johnsen 2014, Marshall et al. 2014). Fortunately, with novel genomics tools, unraveling the molecular components behind thermal acclimation as well as circadian and seasonal rhythms is finally becoming achievable.

1.7. Study species

The group of teleost fish (Teleostei) is the largest group in the class ray-finned fishes (Actinopterygii), and the most species-rich vertebrate group with roughly 27 000 species (Nelson, 2006). Many teleost species are farmed for human food production or used as models for developmental and evolutionary biology. The three-spined stickleback (*Gasterosteus aculeatus* L.) is a small teleost fish from the family Gasterosteidae, distributed around the Northern Hemisphere (Fig. 5). It has experienced repeated adaptive radiations from the saline environment to fresh water, as several populations were confined to lakes following the retracting ice after the last glaciation *ca*. 10 000 years ago. Sticklebacks have become a model for behavioral ecology and genetics (Wund et al. 2015), ecotoxicology (Andersson et al. 2010, Katsiadaki et al. 2010) and evolutionary genetics (Viitaniemi and Leder 2011, Leinonen et al. 2012, McCairns and Bernatchez 2012), and their genome was sequenced by the BROAD institute in 2007. The success of the stickleback as a model species for several research fields has also been facilitated by the ease of maintaining and breeding them in laboratory conditions and being able to catch the fish from different habitats. Moreover, three-spined stickleback is a great model species for ecotoxicologists due to its annual breeding cycle, similar to the majority of teleost fish in human-impacted habitats.

The Arctic char (*Salvelinus alpinus* L.) is a cold-adapted salmonid and the world's northernmost freshwater species (Fig. 5). Its preferred temperatures are in the range of 10–12°C (Jensen et al. 2014, Siikavuopio et al. 2014). As with the stickleback, the Arctic char are found in diverged life-history (anadromous, resident) and morphological forms (Skoglund et al. 2015). Its distribution is circumpolar, but confined to the Arctic, with the exception of few land-locked populations found south of the Arctic Circle. Many of the lake populations have become extremely endangered. In Vuoksi water system in Eastern Finland the Arctic char was present in almost a 4500 km^2 area at the end of the 19th century, but presently viable stocks remain only in a 79 km^2 area (Hyytinen et al. 2006). The cold and seasonal habitat of the Arctic char explains its higher hypoxia tolerance but lower temperature tolerance compared to the Atlantic salmon (Anttila et al. 2015). The adaptive radiation of Arctic char to various cold environments makes it an interesting target for research on the potential adaptations of Arctic species during the accelerating climate warming in the next decades.

Figure 5. Study species, three-spined stickleback (left, photo by Oskari Härmä) and Arctic char (right, photo courtesy of Natural Resources Institute Finland (Luke)).

1.8. Aims of the thesis

This thesis aims at characterizing environmental responses in fish from the levels of individual enzymes to the transcriptome as a whole, in order to better understand the effects of on-going environmental changes on natural populations. The questions I ask in each study are the following:

I. How do metabolic responses to hypoxia and diclofenac interact in the liver? How do circadian rhythms affect the metabolic responses? How do hypoxia and diclofenac affect circadian clock genes?

II. Is diel variation observed in antioxidant enzyme activities? How do antioxidant responses to diclofenac and hypoxia interact in the gills?

III. Which genes and predicted pathways does DBP affect in the testes? Are the responses to a xeno-estrogen and DBP similar?

IV. How does temperature affect diel rhythms in transcription in Arctic char liver? Which genes are robustly affected by temperature?

2. Material and Methods

2.1. Fish husbandry and experimental design

2.1.1. Studies I and II

Three-spined sticklebacks were caught using nets from the freshwater stream Ottersbek in Hamburg, Germany (53.576°N, 9.965°E) in October, 2012. Hence, the age, sex and disease prevalence in the fish was unknown. Fish were acclimated to laboratory conditions (temperature 10°C, light:dark 12:12) for one month prior to the experiment and fed once daily with frozen bloodworms at the start of the light period.

After acclimation period, the fish were exposed to sodium salt of diclofenac and hypoxia alone or in combination. The exposure level for diclofenac was selected based on previous reports of wastewater effluent and surface water levels and studies focusing on biomarker responses, and was set to 1 µg/L, while the hypoxia exposure level was set to a relatively severe 2.0 ± 0.2 mg/L level, which corresponds to approximately 10 % of atmospheric oxygen level. A previous study has shown a northern Baltic Sea population of three-spined sticklebacks to be moderately sensitive to hypoxia with pronounced effects at approximately 24 % air-saturation level (Leveelahti et al. 2011).

The duration of diclofenac exposure was 14 d. On the last day of diclofenac exposure, half of the diclofenac-exposed fish were additionally exposed to hypoxia. A separate group of fish was also exposed to hypoxia without previous diclofenac exposure. During the hypoxia exposures and the last day of diclofenac exposure, nine fish from each treatment were sampled at three time points: 6, 11 and 24 hours after the onset of light period (indicated by ZT6, ZT11 and ZT24). Simultaneously, six fish from no-treatment control tanks were sampled at each time point. The liver and gill tissues were divided in half, and one half of each was stored in RNAlater for gene expression analyses, and the other half snap frozen on dry ice for the enzyme assays.

2.1.2. Study III

Nine-month-old laboratory-reared three-spined sticklebacks were maintained in 10 L tanks, 10 individuals (mixed sex) per tank (18 ± 1°C, L:D 12:12). Fish had been bred in the laboratory over 13 generations, eliminating environmental complications such as unknown chemical exposures or disease presence. The fish were exposed to two endocrine disrupters, EE2 and DBP, at single nominal concentrations of 40 ng/L for EE2 and 35 µg/L for DBP. The concentrations were selected at the high-end of reported environmental concentrations for two reasons: first, to be confident that the EE2 level would be high enough to cause inhibition of steroidogenesis during the four-day exposure, and second, to use a concentration where previous studies had shown potential anti-androgenic effects by DBP in fish. Each compound was diluted using 0.08 mg/L dimethyl sulfoxide (DMSO), hence, as the control group we used a solvent control group exposed only to the same level of DMSO as the treatments. After the four-day exposure, fish were anesthetized, measured, weighed, sexed, and their gonads dissected and snap frozen in liquid nitrogen. The experiment took place at Cefas laboratory, Weymouth, UK, in January 2013. The fish were not in reproductive state during the experiment.

2.1.3. Study IV

Arctic char juveniles (1 year-old) were collected from the aquaculture facility of Natural Resources Institute in Enonkoski, Finland, at the beginning of July 2013. Fish were divided into two flow-through tanks in a similar size-distribution. One of the tanks was gradually heated to 15°C over the course of seven days from the ambient temperature that was *ca*. 8°C, after which the temperatures and oxygen levels were maintained constant for one month. After the acclimation period, fish from each temperature were sampled for liver tissue at three time points during the day: middle of the light period at 12:30, prior to the dark period at 20:30 and at the middle of dark period at 01:30, hereafter referred to as day, evening and night. These time points were chosen because dusk is known to be associated to major transcriptional changes in other species (Doherty and Kay 2010).

2.2. Gene expression analyses

2.2.1. RNA extraction

In studies I and II, the tissue samples were stored submerged in RNAlater at −20°C prior to RNA extraction. Liver tissue RNA was extracted at the University of Turku, while gill RNA was extracted at Hamburg University. From liver tissue, approximately 3−5 mg piece was homogenized with a plastic pestle in Tri Reagent (Molecular Research Center). RNA was isolated with bromochloropropane according to the Tri Reagent manufacturer's protocol, after which the extraction was finished with RNeasy Micro Kit (Qiagen, Austin, Texas*),* including an on-column DNase I treatment. The gill RNA was isolated using the peqGOLD TRIfastTM kit (Peqlab, Erlangen, Germany) in combination with the innuPREP RNA Mini kit (Analytic Jena, Jena, Germany), including an on-column DNase treatment. RNA was quantified using a NanoDrop 1000 spectrophotometer. RNA integrity was confirmed using agarose gel electrophoresis (gills) or 2100 Bioanalyzer by Agilent (liver). Complementary DNA (cDNA) was synthesized from 1 μg of RNA. The cDNA samples were stored at −20°C until used in qPCR.

In studies III and IV, RNA was extracted from flash-frozen tissue using Tri-reagent following the manufacturer's protocol (Sigma Aldrich and Molecular Research Center), including a DNase I treatment. The tissues were homogenized using TissueLyser at 30 Hz speed. RNA was quantified using NanoDrop 1000 or NanoDrop 2000 spectrophotometer and its integrity confirmed using 2100 Bioanalyzer.

2.2.2. Measuring candidate gene mRNA levels using qPCR

The target genes in study I included two genes from core circadian clock (*per1* and *clock*), two genes associated to chemical detoxification (*cyp1a* and *ahr*) and two genes expected to be hypoxia responsive (*ldha* and *eno*). 18S ribosomal RNA gene (*18S*) was used as a reference gene. Primers and fluorescent probes spanning at least one intronic junction were designed to be specific for each gene of interest. qPCR was performed at the Centre of Biotechnology in the University of Turku using a QuantStudio 12 K Flex Real-Time PCR system (ThermoFisher Scientific, Waltham, MA, USA). Raw fluorescence values were grouped by genes and analyzed using LinRegPCR software (Ruijter et al. 2009, Tuomi et al. 2010, Ruijter et al. 2013).

In study II, the qPCR assays were performed in Hamburg University. The assays were done on a total of fifteen genes, which are putatively involved in either the diclofenac metabolism, antioxidant defense or hypoxia response. *Ribosomal protein large P0-like protein* (*Rplp0*) was included as a reference gene. All products were verified by sequencing using a commercial service (GATC, Konstanz, Germany). The cDNA products were amplified in triplicates run on 96-well microplates using an ABI 7500 Real-Time PCR system (Applied Biosystems, Darmstadt, Germany). The specificity of primer and amplification was evaluated using dissociation curves with a temperature range from 60°C to 95°C. Absolute mRNA copies were calculated with the 7500 System Sequence Detection Software 2.0.6 by means of the standard curve method by using 10-fold dilutions (10^8 to 10^2) of recombinant plasmids.

2.2.3. Tissue homogenization and enzyme activity assays

Flash-frozen liver and gill tissues were homogenized in a buffer with 100 mM KH_2PO_4, 150 mM KCl, pH 7.4, using a TissueLyser (Qiagen, Austin, USA). Homogenate protein concentration was measured following the Bradford method (Bradford 1976), using BioRad stock diluted with dH_2O (1:5) and standards prepared from bovine serum albumin (1 mg/mL). Measurements were made at 595nm using an EnVision 2103 Multilaber Reader (PerkinElmer, Wallac, Turku, Finland).

EROD (Ethoxy-resorufin O-de-ethylase) activity is an assay of CYP1A enzyme activity, measuring the efficiency of conversion of the CYP1A substrate 7-ethoxy resorufin to resorufin, which can be detected with a fluorometer (Andersson and Förlin 1992). The assay was performed according to Burke and Mayer (1974), with adaptations for a 384-well microplate.

LDH enzyme, responsible for the conversion of pyruvate and NADH to NAD+ and lactate, was expected to have higher activity during hypoxia exposure, as decreased oxygen availability leads to decreased incorporation of pyruvate into the citric acid cycle and increased conversion to lactate. LDH activity was measured following the protocol of Dalziel et al. (2012) measuring NADH absorbance at 340 nm with adaptations for a 384-well microplate. Conversion rate was detected for three replicates of each sample and subtracted from the conversion rate of a control well without pyruvate (Dalziel et al. 2012).

The activities of antioxidant enzymes were analyzed in triplicates on either 96- (CAT) or 384-well (SOD, GPx) microplates using commercially available kits: SOD assay kit-WST, Catalase Assay kit and Glutathione peroxidase cellular activity assay kit (Sigma-Aldrich, St. Louis, USA). Only gill samples were measured for antioxidant activities. Sample and reagent volumes of the kits were adjusted for 96- or 384-well microplates.

2.2.4. Preparation of microarrays from testis RNA

The microarray analysis of extracted RNA samples was conducted at the Centre of Biotechnology, University of Turku. The RNA samples from testis tissue were amplified and labeled with Cy3 or Cy5 dyes. On each Agilent custom array, modified from Leder et al. (2009), 300 ng of Cy3 and Cy5 labeled sample were hybridized together. To account for the dye-bias caused by differential binding of the dyes, equal numbers of solvent control and each treatment group samples were hybridized with each dye. We used the same solvent control individuals in comparisons to both EE2 and DBP treatments to minimize variation due to individual differences in the controls. Solvent control and EE2 or DBP exposed samples were run on two different chips each carrying eight hybridizations.

2.2.5. Library preparation and RNA sequencing using Illumina HiSeq

Library preparation and sequencing were conducted at the Beijing Genomics Institute (BGI, Hong Kong) using TruSeq RNA Sample Prep Kit v2 (Illumina, San Diego, CA, USA). To perform strand-specific sequencing, the protocol included digestion of the second strand of cDNA using Uracil-N-Glycosylase enzyme. The libraries were assessed for quality and quantity using two methods: checking the distribution of the fragments size using the Agilent

2100 bioanalyzer instrument (Agilent DNA 1000 Reagents) and quantifying the library using qPCR with a TaqMan Probe. Prior to sequencing, all samples were pooled and distributed across four lanes. Additional sequencing for the assembly construction was performed from the same samples pooled across two lanes.

2.3. Statistical and bioinformatic analyses

2.3.1. Studies I and II

For the relative abundance of target genes compared to the reference genes and the activities of measured enzymes, the significance of differences between control and treatment groups and between time points was analyzed *via* linear mixed-effects modeling using Markov Chain Monte Carlo Sampler for Generalized Linear Mixed Model (MCMCglmm) using default priors (Hadfield 2010) in R (R Core Team). Based on MCMCglmm, the predicted means and interquartile ranges of each response variable were calculated after conditioning for the random effects. Probability (p) values were derived by profiling the distributions of a 1000 posterior estimates sampled from the Markov Chain —in all models we used control group sampled at ZT6 time point (middle of light period) as the default contrast. Statistical significance was defined as $p<0.05$. Models were checked for mixing and convergence by visually inspecting trace and density plots, as well as autocorrelation plots of random effects. The results were visualized using package ggplot2 (Wickham 2009).

2.3.2. Study III

The results, extracted using Agilent's Feature extraction software, were analyzed using limma package (Smyth 2005, Ritchie et al. 2015) in R, version 3.1.0. Data were filtered from low-expression probes and technical outliers, and normalized using cyclic-loess normalization. Differentially expressed genes were analyzed via linear model, including normalization factors for the dye-bias of probes and differences in array quality. We defined differential expression with quality criteria (absolute fold-change ≥1.5) combined to statistical significance based on uncorrected p-values ($p<0.05$). This approach was selected on the basis that hardly any genes could be selected as differentially expressed when using the p-values after multiple correction, and since previous reports have shown high false-negative rates in such analysis for microarray data (Cole et al. 2003). Moreover, for a successful exploratory analysis it is necessary to find all genes showing putatively differential expression in each treatment.

Previously collected annotations were used for the microarray probes and supplemented by a human-stickleback comparison of peptide sequences using BLASTp. Out of the 21 000 probes on the array, human ortholog Entrez IDs were available for 17 842. Since not all differentially expressed (DE) genes could be annotated, and the teleost-specific genome duplication has doubled the number of many paralogous genes in the stickleback, we searched for annotations for the missing DE genes manually by using the probes Ensembl IDs. When available, the human Entrez IDs were used in an enrichment analysis of the DE genes, to define whether there were Gene Ontology terms (GO terms) significantly enriched in the lists of up- and downregulated genes in each treatment. Importantly, the enrichment analysis assumes the functions of human and stickleback gene products are conserved, which is known generally to be the case for many conserved genes (Ashburner et al. 2000). The enrichment analysis was performed with ClueGO (Bindea et al. 2009), a plugin for Cytoscape software, which groups the enriched GO terms based on similarity using Kappa score, whereby it is possible to examine the GO terms sharing similar sets of genes, which may encode proteins with similar functions.

2.3.3. Study IV

Since no genome sequence was available for Arctic char, a *de novo* transcriptome assembly was generated from the quality-trimmed paired-end (PE) reads. Due to challenges posed by accurately determining transcripts in a polymorphic, pseudotetraploid species, the transcriptome assembly was built in three steps. At first, the data from all individuals was combined and assembled using Trinity software. The reads were aligned back to the transcriptome using Bowtie2 allowing for a maximum of 40 multiple alignments when no unique alignment could be determined, and the alignment files analyzed with Corset software, which estimates the read counts at the gene level (Davidson and Oshlack 2014). Corset estimates gene abundances based on sequence and expression level similarity, filtering out low-count transcripts that were likely artifacts in the *de novo* assembly.

The read counts per cluster -data were used to determine which samples covered the most variation in expression levels by determining the distance of samples based on all transcripts using DeSeq2 package in R. The distance was largest between samples from cold acclimation and evening time point in the warm acclimated fish. Thus, the samples with the highest number of reads from each of these groups were chosen for the generation of the final assembly. Reads from the selected two samples were run with Trinity. Alignment of the two samples to the assembly revealed that the ratios of unique and multiple aligned reads were similar to the first *de novo* assembly.

To improve the completeness of the assembly, the reads that were not concordantly aligned were used to generate a third assembly using Trinity. Finally, before the third assembly was combined to the second assembly, transcripts generated by both of these assemblies were filtered after a BLAST search between them to reduce redundancy. The transcripts from third assembly that had less than 98% similarity to those present in the second assembly and were longer than 100 bp were appended to the second assembly, thus producing a final *de novo* assembly with 209 537 transcripts. The trimmed PE reads from all samples were then aligned against the final assembly using Bowtie2, and the alignments analyzed for gene-level counts with Corset.

The counts data generated by Corset was used in a DE analysis to find genes showing temporal and temperature-induced changes in mRNA levels. The packages edgeR and limma in R (Smyth 2005, Ritchie et al. 2015) were used for the DE analysis. Transcript clusters showing negligible expression were excluded based on criteria of >3 counts per million reads in at least three samples, and data normalized per library sizes. The voom method was applied to obtain precision weight for mean-variance relationship of cluster expression levels (Law et al. 2014) and the weights included in limma empirical Bayes analysis pipeline.

Differences in read counts were tested with a contrast matrix, including the contrasts of: time points within temperatures, temperatures within time points, the average temperature effect and the interactions between time points across temperatures. The p-values were corrected for multiple corrections in each contrast using Benjamini-Hochberg method, and adjusted p-value <0.01 used as a significance criteria.

Transcripts of genes defined as expressed were included in the annotated transcriptome assembly. Predicted open reading frames' (ORFs) peptide sequences were obtained using Transdecoder software, and annotated using a stepwise procedure: all sequences were annotated with a reciprocal BLASTp hit approach using e-value cutoff $1x10^{-5}$, first with proteins from zebrafish genome (downloaded from Ensembl) and second with proteins from salmon genome (NCBI *Salmo salar* Annotation Release 100). Finally the remaining un-annotated ORFs were annotated with NCBI non-redundant protein database, which contains single copies of unique protein sequences across species. Only annotations below e-value cutoff $1x10^{-5}$ and with >50% length match to the database proteins were included in the transcriptome annotation.

3. Main Results and Discussion

3.1. Diel variation in responses to diclofenac and hypoxia in the liver and gills (I & II)

The effects of different environmental changes on organisms are often studied in isolation, despite the fact that multiple, unrelated stressors are present simultaneously in the natural environment. The occurrence of concurrent hypoxia and pollution is likely in human-impacted environments that can be both eutrophic and receive wastewater effluent, where NSAIDs are among the most commonly detected pharmaceuticals. In particular diclofenac has been shown to accumulate in the tissues of wild and experimentally exposed fish indicating it can have persistent effects on the health of wild populations (Schwaiger et al. 2004, Brown et al. 2007, Mehinto et al. 2010, Brozinski et al. 2013). Thus, this experiment studied the effects of both diclofenac and hypoxia on metabolic responses in the liver and on antioxidant responses in the gills.

In the liver, which metabolizes the majority of xenobiotic substances, co-exposure to diclofenac and hypoxia increased the activity of the detoxification enzyme CYP1A, as measured by EROD activity, at the first and second time point during hypoxia (Fig. 6b). Temporal variation in CYP1A activity was also observed in the control fish, which had higher activity in the morning than in the day.

In a previous study using rainbow trout, an especially sensitive species, diclofenac was found to induce *cyp1a* transcription in both gills and liver (Mehinto et al. 2010), which was partly confirmed in the liver, where all treatments, most of all hypoxia, induced *cyp1a* transcription at ZT11. In the gills, there was a significant decrease in *cyp1a* by co-exposure, insignificant decrease by hypoxia and no effect by diclofenac, which suggests decreased DNA binding of AHR in the gills took place mostly due to the hypoxia treatment. Hypoxic inhibition of AHR activity has also been described in a previous *in vitro* study (Nie et al. 2001), while hypoxic induction of *cyp1a* has been shown in the liver of three-spined sticleback (Leveelahti et al. 2011). Thus, depending on tissue, AHR can be either inhibited or activated by hypoxia. The weak induction of *cyp1a* by diclofenac in the liver can indicate that diclofenac is a weak agonist for AHR, or AHR is not sensitive to low levels of exposure to NSAIDs.

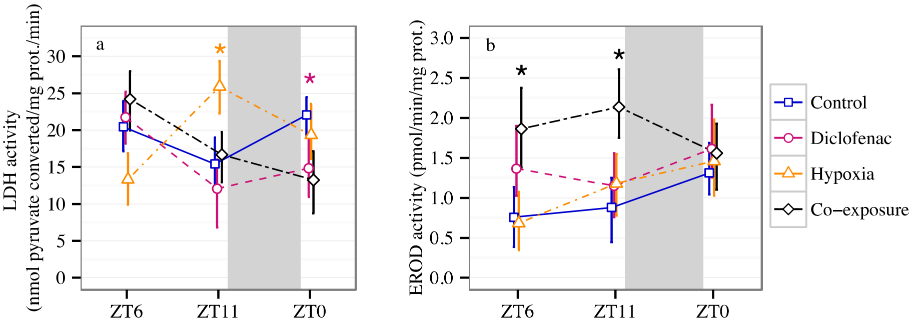

Figure 6. Effects of diclofenac, hypoxia and co-exposure to both on LDH activity (a) and EROD activity (b) in three-spined sticklebacks. Liver tissue samples were taken at zeitgeber time (ZT) 13:30 (ZT6, 5.5 h hypoxia), 18:00 (ZT11, 10.5 h hypoxia), and 7:30 (ZT0, 24 h hypoxia). The dark phase from 19:00 to 7:00 is shaded in grey. Values are means conditioned by random effects ± interquartile range, n = 5–13 fish per treatment and time point in EROD activity and n = 6–10 (except control ZT0 n = 18) in LDH activity. Significant differences between treatments and control within each time point are indicated by * (p = 0.01–0.05), colours indicating treatments.

Similar to CYP1A activity, responses of the anaerobic enzyme LDH to hypoxia were time-dependent. In the liver, its activity was increased after 10.5 h of hypoxia compared to control, but not in the other time points (Fig. 6a). The lack of a similar response in the co-exposure treatment suggests a disturbance of hypoxia response due to diclofenac exposure. This finding was supported by decreased LDH activity in the diclofenac treatment compared to control in the morning (Fig. 6a). In addition, diclofenac has been found to inhibit LDH activity in murine tumor cells (Chirasani et al. 2013). Such an effect may negatively affect wild populations in polluted, eutrophic environments. However, contrasting effects were observed in the gills, where diclofenac and co-exposure treatments increased LDH activity between ZT6 and ZT11, which may indicate a disruption in the ability of gills to uptake oxygen.

Another goal of study I was to measure the effects of diclofenac and hypoxia exposures on circadian clock gene expression. The circadian clock transcriptional loop optimizes the timing of tissue-specific functions. In mammals, disruptions in circadian clock are associated with carcinogenic effects and advanced ageing (Yu and Weaver 2011), but circadian rhythms have not been studied in relation to fitness in wild populations, and little is known of their responsiveness to environmental stress. Transcription in the circadian clock genes *clock* and *per1* was increased by all treatments at ZT11, and decreased in *per1* by hypoxia at ZT0. In the control fish, both *clock* and *per1* transcription decreased from ZT6 to ZT11, but this was not evident in the other treatments. A previous study has demonstrated an interaction between HIF-1 and PER1 in zebrafish, where HIF-1 was able to bind directly to the E-box elements in the *per1* promoter region (Egg et al. 2013). Since transcription in the HIF-1 target gene *ldh* was also increased from ZT6 to ZT11, it is possible that HIF-1 was responsible for the dampened oscillations of *per1* in hypoxia and co-exposure treatments. How diclofenac treatment caused a higher increase than hypoxia in both *clock* and *per1* transcription compared to control is not well understood. One explanation for the effect may have been revealed by the antioxidant enzyme activities measured in the gill tissue. Gill GPx activity was significantly increased between ZT6 and ZT11 in the diclofenac treatment, which suggests increased production of ROS during this time period (Fig. 7). Since circadian clock genes are able to respond to and regulate cellular ROS level (Hirayama et al. 2007, Gyoengyoesi et al. 2013), it remains a possibility that if ROS accumulation occurred also in the liver, it may have contributed to the regulation of *clock* and *per1* transcription.

CAT activity increased in hypoxia and in the co-exposure treatment from ZT6 to ZT11, which supports the GPx results regarding ROS accumulation during this time period. SOD, on the other hand, showed no response to any of the treatments at any time point, suggesting its activity is constitutively high and sufficient to deal with a possible increase in superoxide anion. Overall, the antioxidant responses in gill tissue are compatible with other studies demonstrating that multiple enzymes often do not respond to increased ROS simultaneously, but may instead compensate for each other, as suggested by Leveelahti et al. (2014).

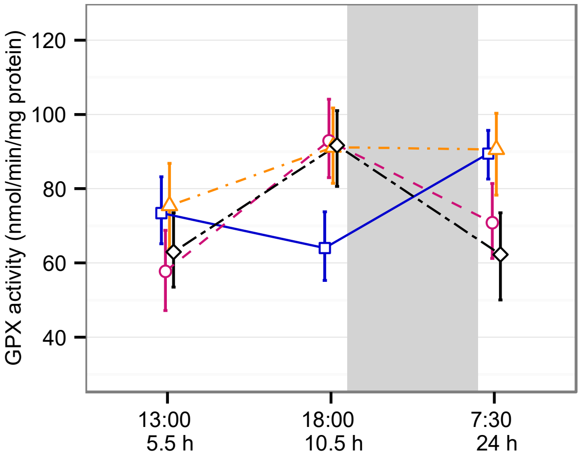

Figure 7. The effects of diclofenac and hypoxia on GPx activity in three-spined stickleback gills. Gill tissue samples were taken at 13:00 (5.5 h hypoxia), 18:00 (10.5 h hypoxia), and 7:30 (24 h hypoxia). The dark phase from 19:00 to 7:00 is shaded in grey. Values are means conditioned by random effects ± interquartile range. Differences between 13:00 and 18:00 in diclofenac and co-exposure treatments were significant (p<0.05). Treatment symbols and colours explained in Figure 6.

Transcriptional responses to hypoxia and diclofenac in the gills at ZT0 were modest, with many genes having high individual variation. However, diclofenac and hypoxia exposures decreased transcription in *cox2*, which supports the hypothesis of conserved responses to diclofenac in humans and fish. *Cox1* and *cox2* inhibition by diclofenac has been described in rainbow trout (Mehinto et al. 2010), but in the present study *cox1* transcription was not affected.

The effects observed at the enzyme activity level were partly in agreement with those observed at the mRNA level. In the liver, CYP1A activity was induced in the co-exposure treatment before its transcription was affected, but by ZT11, both were increased. In contrast, the decrease in LDH activity by diclofenac at ZT0 was not observed at the mRNA level. In the gills, mRNA data were only available for ZT0, where GPx activity tended to have similar responses as the mRNA, and neither the mRNA level nor the enzyme activity of LDH, CAT and SOD changed significantly. Nevertheless, the results obtained from the liver indicate that post-transcriptional modifications can be used to rapidly enhance enzyme activity when environmental conditions change unexpectedly. The responses in liver and gills were highly tissue-specific, but disruptions in either tissue can have systemic negative effects on fish; liver is important not only for detoxifying xenobiotic compounds, but also as an energy storage, fueling both anaerobic and aerobic metabolism, while gills are the main site for gas and ion exchange and acid-base regulation in fish (Perry 1997).

3.2. Distinct transcriptional responses to endocrine disrupters in the testes (III)

Head kidneys and gonads are important sites for steroid hormone synthesis in fish and therefore likely to be involved in endocrine disruption by effluent chemicals. However, the anti-androgenic effects induced by phthalate ester DBP in males are poorly understood. Thus, in this study the genome-wide transcriptional effects of DBP in fish testes were investigated after a short, 4-d exposure, and compared to the effects of a xeno-estrogen, EE2. DBP did not affect similar genes as EE2, and is therefore unlikely to act as a xeno-estrogen. Overall, the effects of DBP were small compared to those of EE2, which caused increased or decreased expression in nearly 200 genes in three-spined stickleback testes at >1.5 absolute fold-change, while DBP affected transcription, mostly decreasing it, in 72 genes. The GO terms that were significantly enriched among genes downregulated by DBP in comparison to all annotated genes present on the microarray are illustrated in Fig. 8. Based on the results, DBP is expected to have potentially negative effects on retinoid metabolism, creatine kinase activity and cell adhesion in fish testes. Earlier studies have reported DBP to induce morphological alterations and abnormal sperm cell aggregation in rodents (Alam et al. 2010, Jobling et al.

2011). Disrupted expression in genes associated with cell adhesion mediated by integrin pathway could contribute to the abnormalities. Likewise, sperm motility and concentration may be affected by genes associated with creatine kinase pathway, as similar effects were found in the mummichog, *F. heteroclitus* by exposure to tributyltin (Mochida et al. 2007).

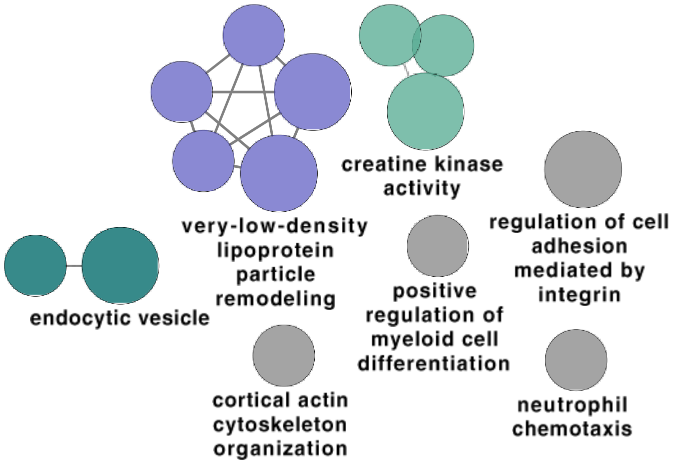

Figure 8. Network of significantly enriched GO terms in genes decreased by 4-d exposure to DBP in three-spined stickleback testes. GO terms sharing similar genes are connected by similar colors and lines, and the most significant terms are named. The size of the circles refers to statistical significance of the enrichment, larger = more significant.

The microarray analysis gave new insight into the potentially fish-specific effects of EE2 and DBP. DBP increased expression in *cytochrome P450 17a2* (*cyp17a2*), which is a paralog of *cytochrome P450 17a1* not found in mammals, by more than four-fold. The protein encoded by *cyp17a2* has previously been shown to possess 17α-hydroxylase but not 17α-lyase activity in medaka (*Oryzias latipes*) and tilapia (*Oreochromis* spp.) (Zhou et al. 2007a). In these species, CYP17A2 is expected to be important for cortisol synthesis and maturation of oocytes (Zhou et al. 2007a, Zhou et al. 2007b). Increased activity in the enzyme is thus expected to increase cortisol synthesis in fish exposed to DBP. In addition, expression in *FK506 binding protein 5* (*fkbp5,* also known as *fkbp51*) was decreased by the exposure to EE2 and DBP, although the effect was smaller in EE2-treated fish (on average 30% vs. 60% decrease compared to solvent control). In humans, FKBP5 functions as a glucocorticoid-induced negative regulator of the glucocorticoid receptor as well as an inhibitor of a variety of other steroid receptors, with the exception of the androgen receptor, which activity it can enhance (Stechschulte and Sanchez 2011). Due to all the connections FKBP5 has to multiple pathways and the finding that its expression can be inhibited by endocrine disrupters, it is relevant to investigate its potential role in endocrine signaling in fish further.

In EE2-exposed fish, as expected, expression in multiple genes important for steroid biosynthesis in the gonads was decreased, including *cytochrome P450 17a1* and *cytochrome P450 11a1, hydroxy-delta-5- steroid dehydrogenase, 3 beta- and steroid delta-isomerase 2* and *steroidogenic acute regulatory protein*. Decreases in the synthesis of these enzymes could lead to inhibition of several steps in steroid biosynthesis, including the transfer of cholesterol over the mitochondrial membranes, the conversion of cholesterol to pregnenolone, the conversion of pregnenolone to progesterone and the conversion of pregnenolone and progesterone to androstenedione (Hsu et al. 2009, Kocerha et al. 2010, Tokarz et al. 2013). Notably, very low overall expression was observed in *hydroxysteroid (17- beta) dehydrogenase 3*, which is a key enzyme in testosterone synthesis in mammals, but has slightly differentiated roles in zebrafish with higher expression in the liver than in the testis (Mindnich et al. 2005).

In summary, transcriptional responses were observed after 4-d exposures to EE2 and DBP at concentrations that were higher than most measured environmental levels but can be encountered by populations inhabiting areas in the proximity of wastewater release sites. The treatments revealed a number of genes that had no previous record of expression in the testes of fish were regulated, thereby supporting future research in the reproductive biology of fish. Since the effects of chemicals on organisms are dependent on several factors, including the studied species, tissue type, developmental stage, and the concentration and duration of the exposure (Lam and Gray 2001), emphasis on future studies should also be on determining a concentration-response curve for DBP at environmentally relevant levels and in chronic exposures. This information will be useful for generating safety limits for DBP exposure in humans and in wild populations. In conclusion, this study adds an important contribution to the understanding of the initial mechanisms by which effluent chemicals can affect reproductive processes in fish.

3.3. Temperature-dependent diel rhythms in transcription in Arctic char (IV)

Circadian clocks regulate transcription of hundreds of genes in numerous species and tissues and mediate the signal of day-length to peripheral clocks *via* the central clock. The mechanism behind the effects of peripheral circadian rhythms on gene regulation in fish, especially in species adapted to highly seasonal variation in photoperiod, is currently poorly understood (Jørgensen and Johnsen 2014). High temperature has been shown to decrease the amplitude of melatonin rhythm, which in turn can delay maturation in salmon (Porter et al. 1999, Porter et al. 2001), but little is known as to how these rhythmic responses are reflected at gene regulation level. Attempting to answer such questions, Arctic char liver tissue transcriptomes were compared between three time points after two temperature acclimations in late summer, under naturally decreasing day-length.

Significant variation in gene expression was found between time points only within the warm acclimation temperature, while no genes showed temporal variation within the cold acclimation temperature. This result was remarkable considering that photoperiod is considered as one of the main zeitgebers of circadian gene expression (Hirota and Fukada 2004). The complete arrhythmia in cold-acclimated fish indicates a lack of light responsiveness and rhythmic endocrine signaling, and not merely decreased amplitude in rhythmic expression. In the wild, Arctic char seek cool temperatures during the summer, but frequently forage also in slightly warmer waters, and in the fish hatchery the rearing temperature is maintained below 13°C throughout the summer (personal communication). Long-day photoperiods are known to initiate preparations for spawning, while short-day photoperiods induce the final maturation of gametes in Arctic char (Jørgensen and Johnsen 2014). The uncoupling of photoperiod signals and gene expression regulation could enable the fish to avoid spawning in conditions when sufficient growth to prepare for reproduction and overwintering is not possible due to low metabolic rate. In agreement with this hypothesis, one of the genes most significantly increased by cold acclimation was the ortholog of *zona pellucida glycoprotein 2*, which is predicted to be associated with increased estrogen levels and thus, inhibition of sexual maturation in male fish (Westerlund et al. 2001, Genovese et al. 2012).

Within the warm acclimation, samples collected during the day were more similar to samples collected at night than to samples collected in the evening (Fig. 9). The direction of change from day to evening or night was for the most part increased expression with higher than two-fold change. The larger differences between day and evening samples than between the day and night samples suggest that the physiological changes associated with the dark period are more likely to be shown at the transcriptional level prior to the dark period than during it. Previous studies have also found the preparation for the dark period to be associated with major functional changes in mouse and *Drosophila melanogaster* (Doherty and Kay 2010).

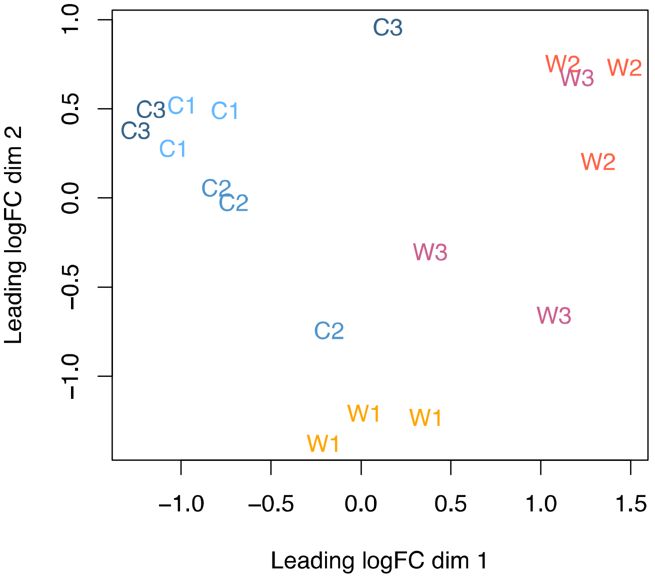

Figure 9. Multidimensional scaling (MDS) plot generated based on 2000 genes with highest pairwise fold-change differences between samples. Samples collected after warm (W, 15°C) and cold (C, 8°C) acclimations at day, evening and night are indicated by 1, 2 or 3, respectively.

A GO analysis revealed most of the genes with increasing expression between the day and the evening to be related to metabolism (Fig. 10). In the statistical overrepresentation analysis, protein methylation was the most significant pathway among genes more expressed in the evening than day with four genes. In addition, seven GO terms related to RNA metabolism and transcription and three GO terms related to translation were overrepresented at a slightly higher p-level. It thus appears that shifts in metabolism and in the transcription-translation machinery are involved in the transition between the light and dark periods. These findings are compatible with the expectation that many of the genes under rhythmic regulation are regulated at epigenetic, transcriptional and translational levels (Harms et al. 2004, Ripperger and Merrow 2011, Kojima et al. 2012).

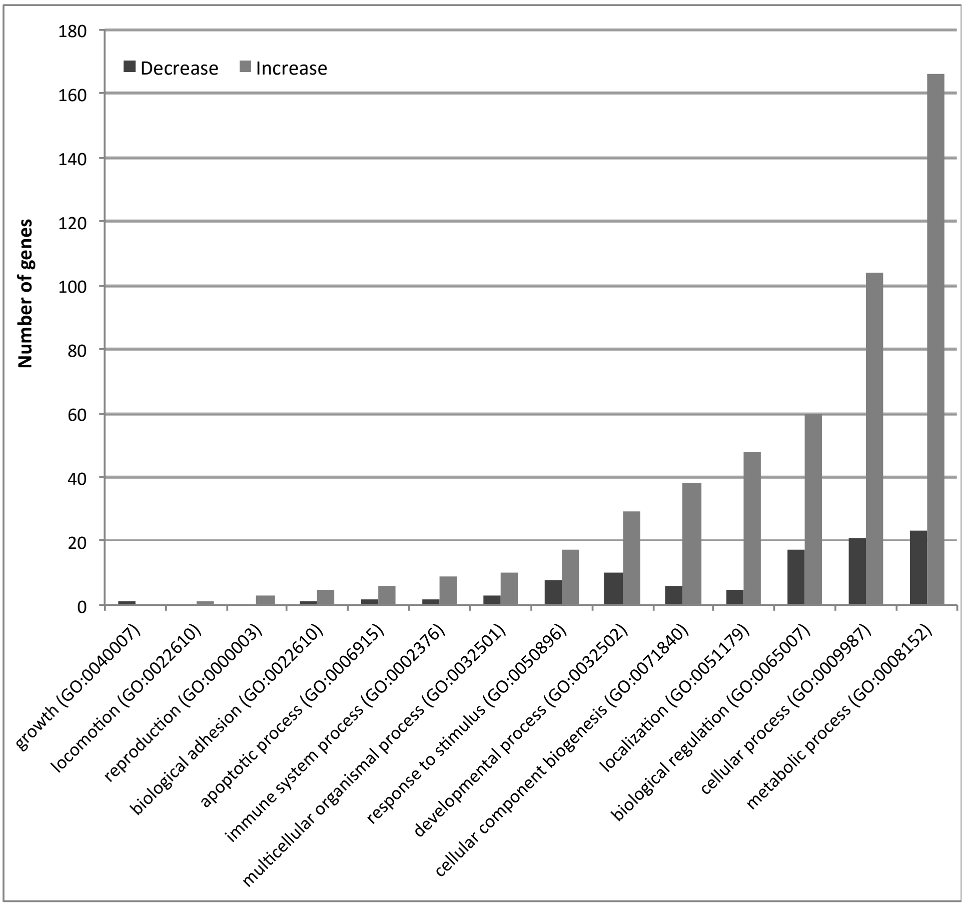

Figure 10. Biological processes associated with zebrafish gene orthologs that were differentially expressed between the day and evening in warm acclimated Arctic char. Many more genes were increased than decreased in the evening compared to the day, most of which were related to metabolic processes.

There were more genes with higher expression in the cold-acclimated fish than in the warm-acclimated fish (2 237 vs. 1 149, respectively). In cold acclimation, transcription in several genes related to cold tolerance and pathways associated to apoptotic processes and lipid metabolism was increased. In contrast, the glycolysis pathway was significantly overrepresented in genes more expressed in the warm-acclimated fish. Together, these results are compatible with the hypothesis that a shift from glucose to lipid metabolism is associated with cold acclimation, which has been previously described in hibernating mammals and teleost fish (Schultz and Conover 1997, Dark 2005). The results also corroborate previous studies suggesting *cold-inducible RNA-binding protein* (*cirbp*) can mediate responses to cold acclimation in fish and mammals (Nishiyama et al. 1997, Gracey et al. 2004, Sano et al. 2015). CIRBP has also been shown to modulate circadian clock gene expression post-transcriptionally in human cells (Morf et al.

2012). Hence, whether CIRBP regulates seasonal variation in clock gene responsiveness to day-length should be investigated further.

The Arctic char is known not only for its sensitivity to high temperatures but also for its remarkable morphological and life-history plasticity (Jørgensen and Johnsen 2014, Kapralova et al. 2015). Since high phenotypic plasticity, the ability of the same genotype to produce multiple phenotypes across different environments, can promote adaptive divergence of populations under changing environmental conditions (Aubin-Horth and Renn 2009, Morris et al. 2014, Dayan et al. 2015), this may allow Arctic char to adapt in novel thermal environments, and the sensitivity of the biological clock to temperature could be one mechanism to achieve this. For instance, temperature regulation could enable the fish to avoid spawning when the temperature is not optimal even though the photoperiod would signal spawning season. The results also suggest that direct temperature regulation of the clock can mediate the impact of abnormal seasonal temperatures and extreme weather events, which may lead to disrupted seasonal changes in the physiology of Arctic fish.

4. Conclusions

In the present era, characterized by human dominance on the planet, abiotic conditions and species composition in ecosystems are changing rapidly. In one hundred years, the natural world will be in many unexpected ways different to the current one. Do humans have the knowledge to secure sustainable food production for the growing population? How can we prevent ecological disasters? Can we establish colonies on other planets? A thorough understanding of biological processes will be instrumental in creating a new relationship with the natural world, one that is not based on exploitation and eradication but knowledge and sustainability. In this thesis I have addressed questions on the impacts of the present-day environmental challenges on gene expression in two species of teleost fish. Since gene expression is the process that links genome information to the physiological response, which in turn modifies behaviour, the results of the thesis can lead to a better understanding of reproduction, biological rhythms and adaptation to climate change in fish.

My results show how the effects of circadian rhythms are visible in numerous genes at both the enzyme and mRNA levels during environmental stress, but also in the absence of stressors. I found the strongest responses to the painkiller diclofenac and hypoxia in both the liver and gill tissue in the evening. Similarly, I observed the majority of thermal effects on transcription occurred in the evening in the liver tissue of Arctic char. Since diel rhythms in environmental responses have been neglected in most ecophysiological studies, it is not known how widespread this phenomenon is, but supporting my findings, chronobiologists have shown that dusk and dawn are associated with large changes in transcription in mammalian and insect model species (Doherty and Kay 2010). This pattern is counterintuitive considering that behavior and physiology can be expected to be more different between night and day than between the evening and the day. However, protein synthesis can be a time-consuming process, and post-transcriptional regulation can be used to adjust its timing differently from the mRNAs' oscillation. Thus far, numerous studies have demonstrated how these mechanisms regulate the timing of protein oscillations (Alvarez-Saavedra et al. 2011, Shende et al. 2011, Xue et al. 2014), and the topic will undoubtedly receive increasing attention in the future. Likewise, the roles of non-coding RNAs in the regulation of hypoxia responses are starting to emerge (Ivan et al. 2008).

The ecological risks associated to human-introduced chemicals in the environment have been taken into consideration since Rachel Carson revealed the correlation between the pesticide dichlorodiphenyl-trichloroethane (DDT) and the loss of bird populations in North America in her novel Silent Spring in 1962. DDT and a number of other compounds have been banned in developed countries and wastewater clean-up is advancing, but the use of chemicals in agriculture and defects in wastewater purification still present serious risks in large parts of the world. Besides finding resources to minimize pollution around the globe, we must try to understand whether the increasing release of pharmaceuticals, plasticizers and other micropollutants in purified wastewater is posing a risk to wildlife. In my thesis, I showed diclofenac, EE2 and DBP have effects on gene expression in fish, while earlier studies have reported their organismal effects (Van den Belt et al. 2001, Mehinto et al. 2010, Saaristo et al. 2010, Chen et al. 2014, Xu et al. 2014) indicating they could decrease fitness or survival of wild populations. Consequently, there is a need to decrease the presence of these compounds in wastewater. Increasing awareness of the public regarding the risks of these substances and implementing new procedures from pharmacies to industry and wastewater treatment will be important means for reaching the goals to this end.

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