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Proteomics Approach for Toxicity Assessment in Zebrafish (*Danio rerio*) Embryos

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2.
3.

Tag der mündlichen Prüfung:

"You see something a hundred times, a thousand times,
before you see it real for the first time."

Christian Morgenstern

General Structure

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List of Abbreviations

AMC	7-Amino-4-methylcoumarin
BSA	Bovine serum albumin
c	Control
Cat	Cathepsin (CatH- CathepsinH, CatC- CathepsinC, CatL- CathepsinL)
CHAPS	3-[(3-Cholamidopropyl)-dimethylammonio]-propan-sulfonat
COX	Cycloxygenases
D	Dimension
Da	Dalton
DarT	*Danio rerio* Teratogenicity Assay (Embryo Test with the *Danio rerio*)
DC	Detergent compatible
2-DE	Two-dimensional gel electrophoresis
DIGE	Difference Gel Electrophoresis
DIN	Deutsches Institut für Normung
DMSO	Dimethyl-sulfoxide
DNOC	4,6-Dinitro-o-cresol
dpf	Days post fertilization
dpi	Dots per Inch
DR	*Danio rerio*
DTT	Dithiothreitol
EC	Effect Concentration
Eq	Equation
EROD	7-Ethoxyresorufin-*o*-deethylase
ESI	Electron Spray Ionisation
GST	Gluthatione S Transferase
hpf	Hours post fertilization
HSP	Heat Shock Protein
ISO	International Organization for Standardization
Lv	Lipovitellin
MALDI	Matrix Assisted Laser Desorption/Ionisation
MOA	*Mode of Action*
MolDarT	Molecular *Danio rerio* Teratogenicity Assay
MS	Mass Spectrometry
MW	Molecular Weight

NSAID	Non Steroidal Anti-Inflammatory Drugs
oe	Over-expressed
OECD	Organisation for Economic Co-operation and Development
PAGE	Polyacrylamide Gel Electrophoresis
PC	Principal Component
PCA	Principal Component Analysis
PES	Protein Expression Signature
PTM	Posttranslational Modification
Pv	Phosvitin
QSAR	Quantitative Structure-Activity Relationship
REACH	Registration, Evaluation and Authorisation of Chemicals
rp	Repressed
RSD	Relative Standard Deviation
RT	Room Temperature
SDS	Sodium Dodecyl Sulfate
SOD	Superoxide Dismutase
U	Unit
V	Volt
Vtg	Vitellogenin
WHO	World Health Organisation
WIK	Wild Type India Kalcutta

CHAPTER 1

General Introduction

The characterisation of responses to toxic exposure at the molecular level of biological systems is a major challenge in ecotoxicology because it enables the unravelling of mechanisms of toxicity, the discovery of novel biochemical biomarkers and early diagnoses of exposure and effects. The unbiased and non-hypothesis-driven approaches of functional genomics techniques, like proteomics, allow simultaneous analyses of large groups of biomolecules. This makes them powerful tools for comprehensive molecular effect screening. But, they have only recently been introduced in the field of ecotoxicology. The extension of these techniques towards well known ecotoxicological model organisms like zebrafish (*Danio rerio*) embryos could offer a high potential for a more thorough understanding of toxicity. With the chance to find novel biomarkers they may also promote rapid and sensitive toxicity screening.

In this introductory chapter the necessities for understanding of molecular toxicity processes and for development of novel biochemical biomarkers are briefly discussed. In addition, proteomics as a molecular effect screening method and the zebrafish as an ecotoxicological model organism are introduced. Finally, the general objectives of the different investigations described in this thesis are presented.

Molecular Effects, Mechanisms of Toxicity and Biochemical Biomarkers

Contaminants affect biological systems at many levels of organisation from molecular to ecosystem levels. However, it is widely accepted, that all toxicant effects begin with biochemical responses by interaction of the toxicants with biomolecules (Clements 2000, Jagoe *et al.* 1996) before cascading through higher organisation levels (Figure 1-1). Consequently, effect analysis at the biochemical level might on the one hand contribute to knowledge about underlying molecular events of toxicity (mechanism of toxicity) and on the other hand enable early and specific prediction of toxicological effects at higher organisation levels (biochemical biomarkers) (Waters and Fostel 2004).

Mechanisms of Toxicity

All underlying molecular events of toxicity and the processes how chemicals exert their toxic effects are comprised as mechanisms of toxicity. Since multiple interactions of the toxicants with biomolecules can be assumed, different levels have to be considered for a comprehensive understanding of toxicity mechanisms (reviewed in Boelsterli 2007). Toxicity mechanisms include mechanisms of uptake, distribution, metabolism and elimination of the chemicals (toxicokinetics), specific or non specific

primary interaction with target molecules (toxicodynamics), effector mechanisms and, as a consequence, information on biological responses (defence, tolerance, repair, etc.). Even if many toxicity mechanisms have still to be unravelled, some toxicity related processes have been well understood e.g. detoxification processes (phase I or phase II metabolisation processes), stress proteins response (heat shock proteins), xenobiotic-induced oxidative stress, DNA modification mechanisms or immune mechanisms.

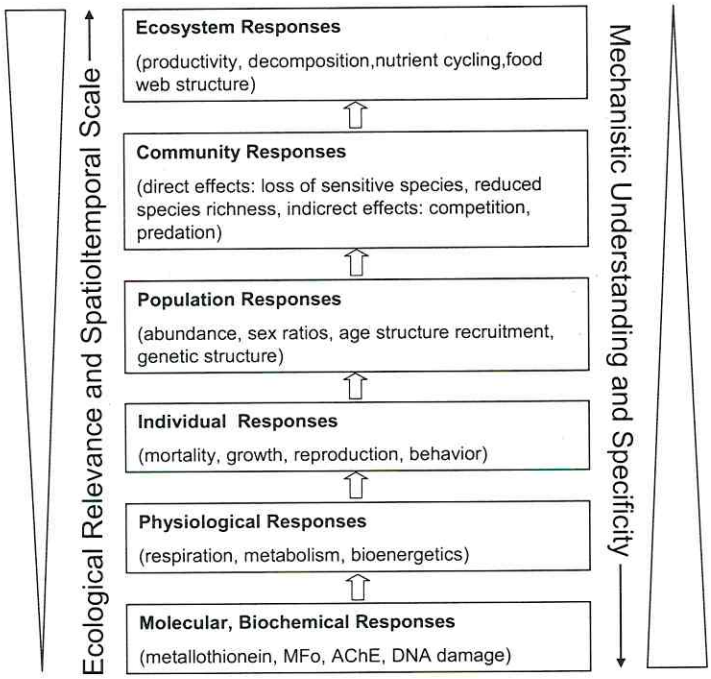


Figure 1-1 Relationship between ecological relevance, spatiotemporal scale, mechanistic understanding and specifity across different levels of organisation. Figure was modified from Clements (2000).

Next to toxicity mechanisms which presume the understanding of detailed molecular events, a more generic term, *Mode of Action* (MOA) has been created. MOA describes the molecular key events and basic processes that lead to structural or functional toxic effects. All characterised molecular events of toxicity hence contribute to the deeper understanding of the MOA and only in conjunction with further studies on these processes may help to identify whole mechanisms of toxicity. The importance for MOA analysis and unravelling of toxicity mechanisms has been highlighted by Boelsterli (2007). As the most compelling reasons for a deeper understanding of mechanisms, Boelsterli (2007) described the ability to better extrapolate data and to improve risk assessment of potentially toxic chemicals for environmental and human safety.

This includes for instance:

- the prediction of effects of untested contaminants basing on similarity of biochemical mode of action to well understood contaminants,
- the support in developing quantitative structure activities relationships (QSAR) (Livingstone 2000)
- the characterisation of high-risk subgroups of chemicals,
- the definition of thresholds exposure levels for toxic compounds,
- the prediction of chronic effects
- or the development of antidotes for prevention of acute intoxications.

So, the elucidation of toxicity mechanisms is one of the most important bridges between hazard identification and risk assessment, leading to toxicity prediction and prevention (Boelsterli 2007).

Biochemical Biomarkers

Next to the deeper understanding of toxicity mechanisms, effect analysis at the molecular level may lead to the discovery of novel biochemical or molecular biomarkers (reviewed by Monsinjon and Knigge 2007, Dowling and Sheehan 2006, Clements 2000). The term 'biomarker' is used in a broad sense to include almost any measurement reflecting an interaction between a biological system and an environmental agent, which may be chemical, physical or biological [World Health Organisation (WHO) 1993]. According to their indications, they are generally subdivided into *biomarkers of exposur*e, *biomarkers of effect* and *biomarkers of susceptibility*. They might be applied to assess the exposure and effects of chemicals or the susceptibility of individuals to respond to exposure of these. The resulting information can be used for health risk assessment, clinical diagnosis or monitoring purposes (WHO 1993). Biochemical or molecular biomarkers are deduced from measurable effects at the biochemical level and offer some benefits in comparison to more classical (physiological, morphological, behaviour related) biomarkers:

- As reviewed by Clements (2000), responses at the molecular level occur more rapidly than at higher organisation levels. Therefore, biochemical biomarkers could provide early warnings for toxicological effects.
- Toxicants change the structural and/or functional properties of multiple biomolecules (Boelsterli 2007). Therefore, Monsinjon and Knigge (2007) described the potential of establishing multiple biomarkers or biomarker pattern

at the molecular level indicating whole affected biochemical pathways (effect patterns) and/or exposure against multiple toxicants.

- Moreover, being directly associated with molecular processes, molecular biomarkers might be very specific for certain substances and also directly hint to mode of actions of the toxicants (Monsinjon and Knigge 2007).
- Stegeman *et al.* (1992) described easy assay methods as important criteria for novel biomarkers. Molecular biomarkers, like enzymes, proteins or gene expression rates, are often very accessible to fast biochemical measurement methods (like enzymatic assays, PCR or Western Blot analyses) and thus may enable high-throughput and time-saving screening assays (Hagger *et al.* 2006, Liedke *et al.* 2008, Muncke *et al.* 2006).

The importance for novel molecular biomarkers is underlined if one considers that many known biochemical biomarkers have been proposed for implication in regulatory toxicology and environmental management (Galloway *et al.* 2004, Handy *et al.* 2003, Hagger *et al.* 2006, Gundert-Remy *et al.* 2005, Moore *et al.* 2004). Several criteria were defined which should be fulfilled by each novel candidate biomarker (Mayer *et al.* 1992, Stegeman *et al.* 1992). These include sensitivity, reliability and variability criteria as well as easy measurement techniques, baseline data and also information on the test organisms. Most of the well established molecular biomarkers are e.g. related to oxidative stress (e.g. superoxide dismutase), detoxification mechanisms (e.g. cytochrome P450, gluthathion-S-transferase), sequestration (e.g. metallothionein), neurotoxicity (e.g. acetylcholinesterase), proteotoxicity (e.g. heatshock proteins) or endocrine disruption (vitellogenins). But the number of known molecular biomarkers is still small and several publications (Boelsterli 2007, Galloway *et al.* 2004, Gundert-Remy *et al.* 2005, Monsinjon and Knigge 2007, Moore *et al.* 2004, Van der Ost 1997) have emphasized the high demand for novel biochemical markers.

Functional Genomics Methods Promise Unbiased Analysis of Multiple Biomolecules

Functional genomics techniques, such as, *genomics, proteomics* or *metabolomics,* allow the simultaneous and unbiased analysis of huge sets of biomolecules like genes, proteins and metabolites (Figure 1-2). During the last decade, these techniques have emerged to the most powerful tools for molecular effect assessment in toxicology. Recently, their importance also in ecotoxicological research has grown and the number

of released functional genomics publications related to ecotoxicology has increased. Within the field of ecotoxicology, functional genomics methods (e.g. genomics, proteomics or metabolomics) are generally summarised as *Ecotoxicogenomics (*Miracle and Ankley 2005, Snape *et al.* 2004*)*.

Boelsterli (2007) has observed that, even if nature is more complex, toxicity mechanisms often tend to be described as single or as a chain of molecular and cellular events to reduce complex pictures to more simplistic models for better understanding (see Figure 1-2). Functional genomics methods provide analysis tools which could capture complex toxicity processes and screen whole patterns of disturbances at the molecular level. Therefore, they hold the potential to contribute to the discovery of multi biomarker panels indicating multiple exposure scenarios or whole affected biochemical pathways besides of the finding of single affected molecules (Monsinjon and Knigge 2007). Moreover, if different functional genomics techniques are applied together within a system biological approach, they could allow the comprehensive understanding of the processes underlying toxic effects.

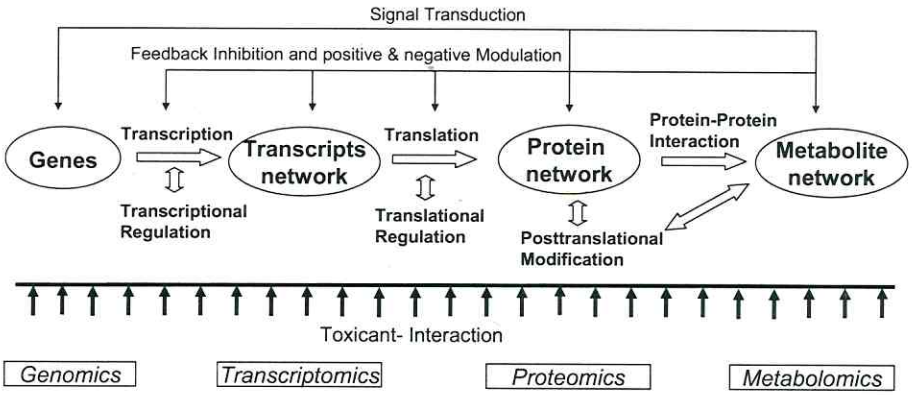


Figure 1-2 Different levels of biomolecules, their inter- and intra-molecular interactions and feedback regulations and possible toxicity targets are indicated. Functional genomics-techniques such as genomics, transcriptomics, proteomics and metabolomics may reveal toxicity caused changes at different molecular levels. The figure was modified after Weckwerth (2003).

Proteomics: Proteomics means the analysis of the global protein profile of a cell, organ or organism, measuring protein expression in relation to time, development and interactions with the environment. The term *"proteome"*, first introduced in 1995 (Wasinger *et al.* 1995), describes the total set of proteins expressed by its genome in a given cell at a given time. Related to toxicity based applications, proteins showing quantitative expression changes after certain chemical exposure are proposed to be implicated in toxicological responses. Applying bioinformatic and identification tools,

they could be related to distinct biochemical pathways and may be considered as candidate biomarkers of certain exposure or effects. To date, some proteins have been described to be implicated in toxic stress events (e.g. cytoskeleton proteins, ATP synthase, proteasome and myosin) or are already established as biomarkers (e.g. superoxide dismutase, heat shock proteins, gluthathione-S-transferase, catalase and cytochrome P450). Proteomics approaches can reveal proteins as being involved in toxicological responses that have not been described previously.

Proteomics is considered as the complementary approach to transcriptome analysis which studies gene expression by analysing all transcripts (mRNAs) in a cell. In comparison to mRNAs, proteins represent the functional units of a cell or tissue and hence, proteome analysis indicates changes that are more accessible to a functional interpretation. This is confirmed by the work of Anderson and Seilhamer (1997) describing a correlation coefficient of only 0.48 between mRNA and protein abundances in human liver. In addition, proteomics integrates post-translational modifications, phosphorylation and dephosphorylation events, which are important for functional status of the proteins and can not be detected by transcriptome analysis.

Graves and Haystead (2002) have distinguished between three different types of proteomics, which they termed as (*i*) expression proteomics (comparison of protein abundance between different conditions), (*ii*) structural proteomics (analysing protein complexes and protein-protein interactions) and (*iii*) functional proteomics (specific directed proteomics approaches). Monsinjon and Knigge (2007) have outlined that expression proteomics is the predominant application within ecotoxicology. The proteomic studies in the present thesis are based on expression proteomics as well.

Two main workflows being associated with expression proteomics (Figure 1-3) are described (Monsinjon and Knigge 2007) and are applied in the present thesis. These workflows are related to data mining processes for finding candidate proteins associated with the toxic response. On the one hand an identity-based approach based on univariate spot-to-spot or peak-to-peak analysis and the identification of proteins with differential expression is described. On the other hand pattern-only approaches based on multivariate proteomic pattern recognition are introduced. These do not aim at protein identification in the first place and mainly strive to discriminate between contaminant exposure and control conditions. For detected changed protein pattern (key proteins) Bradley and coworkers (Shepard *et al.* 2000a and 2000b) coined the term of protein expression signatures (PES). However, the two approaches are not exclusive of each other and

represent two different ways to validate the outcome of proteomics studies (Monsinjon *et al.* 2006).

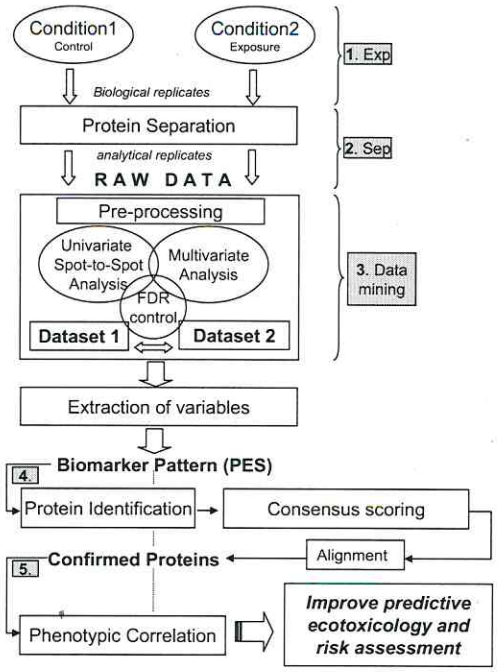


Figure 1-3 Schematic representation of the environmental proteomics workflows for expression proteomics. (1) experimental condition, (2) protein separation, (3) preprocessing and data mining with either univariate spot-to-spot and/or multivariate analysis with false discovery rate (FDR) control, (4) and (5) validation, protein identification, biomarker pattern recognition, correlation with phenotypic data and classical toxicological endpoints (Figure modified from Monsinjon and Knigge (2007)).

Ecotoxicoproteomics generally consists of a multi-parametric workflow procedure beginning with contaminant exposure of the organism followed by the separation of the protein samples and, as one of the last steps, by mostly mass spectrometry (MS) based identification of the proteins of interest (Figure 1-3). Hereby, the protein separation is one of the most important steps towards a successful proteomic approach. Despite of some limitations, the two-dimensional gel electrophoresis (2-DE) technique is still the most sufficient and frequent used protein separation method in quantitative proteomics (Berth *et al.* 2007; Lopez 1999; Rabillourd 2002) and was hence chosen for proteomic analysis in this work. 2-DE combines the two gel-based separation techniques, isoelectric focussing (IEF) and SDS-PAGE, and separates the proteins in two dimensions, first according to their isoelectric point (pI) and second molecular weight (MW). It allows the separation of a high number of proteins, up to 10.000 protein spots on one gel (Klose and Kobalz 1995), the quantitative analysis of the proteins and also

their MS-based identification. Several software tools can be found on the market enabling quantitative analysis of 2-DE data. In this thesis, the 2-DE software package Delta 2D (DECODON) was chosen for data analysis as it allows an appropriate data handling for ecotoxicoproteomics applications (Berth et al. 2007). For identification, the proteins are excised from the 2-DE gels and are cleaved into smaller fragments by trypsin digestion. Subsequently, the protein fragments are analysed and characterised by MS methods. Protein fragments in the present thesis were analysed by Electronspray Ionisation MS (ESI-MS). Protein identification data can subsequently be obtained by correlating the MS data with sequence databases. Hereby the sequence query method, which is based on interpretation of MS/MS spectra and combines peptide molecular masses with amino acid- sequence and - composition information is potentially the most powerful search method compared to others (http://www.matrixscience.com/help/sq_help.html) and was used for protein identification in the present thesis.

Statistical analysis of 2-DE data is mostly limited to univariate methods since pattern analysis require large sample sizes which are not suitable for 2-DE, mostly conducted with few replicates (Meunier *et al.* 2005). However, even if requiring normal distribution of the data which is critical considering the few replicate numbers, the univariate statistical methods for proteomics data analysis are generally based on the Student's *t*-test. This analysis results in a list of proteins whose differential expression is significant at a predefined level, usually $p < 0.05$ ($\alpha = 5$ %) (reviewed in Monsinjon and Knigge 2007). These significances are commonly combined with a greater than two-fold expression change to obtain the final PES (Karp *et al.* 2005, Meunier *et al.* 2005). Univariate methods as well as multivariate pattern analyses were applied for proteomics data mining in this study.

Since 2-DE is the combination of two separate techniques, there are many parameters which could be changed to establish optimised protein separation patterns. These include cell lysis protocols, buffer compositions, loading techniques, IEF and SDS-PAGE concerning parameters as well as protein visualisation techniques. Protein visualisation comprises a large field of different staining techniques ranging from more classical post-gel-staining methods like the sensitive silver staining (Heukeshoven and Dernick 1988) to more recently used fluorescence dye based pre-gel-staining approaches like DIGE (difference-gel-electrophoresis) (Gade *et al.* 2003, Marouga 2005).

For all protein samples investigated in this thesis, the 2-DE parameters were optimised for receiving well separated protein patterns. The applied proteomic methods in the present thesis were based on a 2-DE approach at which proteins were visualised with silver staining due to the high sensitivity and low costs. To ensure successful identification of the proteins with MS based methods, which could interfere with silver staining, a MS compatible silver staining method according to Yan *et al.* (2000) was used.

Zebrafish Embryos as Model Organism in Ecotoxicology

Fish are vertebrate models in many scientific disciplines such as evolution and development biology, medicine and toxicology. On the one hand they are the most numerous and phylogenetically diverse group of vertebrates. On the other hand there are high levels of conservation in genetic programs controlling development, fundamental physiologic processes, tissue types as well as endocrine systems between fish and other vertebrates including mammals. As they are among the highest developed vertebrates in aquatic systems they are also very important in the field of ecotoxicology.

Among fish, the zebrafish (*Danio rerio*), a small tropical fish native to the rivers of India and South Asia (Eaton and Farley 1974), has emerged to one of the best-described and most popular vertebrate model species (Coverdale *et al.* 2004, Nagel 2002, Spitsbergen and Kent 2003) in many scientific fields including ecotoxicology. This is due to many benefits like the rapid development, easy maintenance in laboratory, large number of offspring, transparency of the embryos and access to experimental manipulation. In addition, the sequencing of the zebrafish genome is highly advanced and will be completed soon (http://www.sanger.ac.uk/Projects/D_rerio/). Hence, the zebrafish is unique with respect to the level of available knowledge, technology and approaches and has attracted large-scale funding, e.g. by the European Union (Bradbury 2004).

In ecotoxicology, zebrafish adults are used for acute toxicity testing of chemicals (OECD 1992). Recently, Nagel (2002) has introduced the zebrafish embryo test (DarT, *Danio rerio* teratogenicity assay) as an alternative to the acute fish toxicity test and animal replacement method. The use of fish embryos is not regulated by current legislations on animal welfare and is therefore considered as a refinement, if not replacement of animal experiments. The DarT analyses acute toxicity in 48 hour long exposed embryos by screening lethal effects, developmental disorders and other

morphological, sublethal endpoints, such as changes in heart beat, spontaneous movements and hatching-rate or -time. The development of the DarT was enabled by the high available knowledge of developmental processes in the zebrafish. The rapid development (hatching of embryos at 2-3 days after fertilisation) and the transparent chorion of the zebrafish eggs allow easy observation of development (Kimmel *et al.* 1995). This has contributed to a large extent to the current understanding of development processes and thus, to the application of zebrafish embryos as model organisms in developmental biology (Westerfield 2000).

In Germany, the fish embryo test has already replaced the acute fish tests for toxicity assessment of waste water effluents (Federal Law Gazette 2005, DIN 38415-T6). A first draft for a guideline to extend the fish embryo test also for chemical testing has been submitted to the Organisation for Economic Co-operation and Development (OECD) and is currently under review (OECD 2006). This is especially of high concern considering the new European chemical policy REACH (registration, evaluation and authorisation of chemicals) which leads to a dramatic increase in the number of animal experiments. Hence, there is a great demand for animal replacement methods for ethical reasons. But as the zebrafish embryo assay promise to be less cost-intensive and less time-and space consuming, also industry is interested to promote the embryo assay in ecotoxicological testing.

Next to acute toxicity testing, zebrafish in developmental stages have also experienced many other toxicity related applications which were recently reviewed by Scholz *et al.* (2008). These include approaches leading to a better molecular understanding of toxicity (MOA) including detoxification studies (Wiegand *et al.* 2000), xenobiotic transporter analysis (Leslie *et al.* 2005), endocrine disruption analyses, immune modulations and genotoxicity studies. In addition, several functional genomics studies on zebrafish have been carried out (Coverdale *et al.* 2004,Carney *et al.* 2004, Hoyt *et al.* 2003, Ton *et al.* 2003, Voelker *et al.* 2007, Weil *et al.* 2008, Yang *et al.* 2007, Link *et al.* 2006, Shrader *et al.* 2003). Genomics studies on early life stages of the zebrafish have lead to the extension of the DarT assay to the MolDarT, based on testing of molecular effects in the transcriptomic toxicity range which could e.g. be used for the indication of chronic toxicity (Liedtke *et al.* 2007, Muncke and Eggen 2006, Muncke *et al.* 2007, Voelker *et al.* 2007). The MolDarT is only one of the examples showing the high potential for the application of zebrafish in early life stages in molecular effect screening.

Objectives

The overall objective of the present thesis was to develop and characterise a ecotoxicoproteomics approach with early life stages of the zebrafish (*Danio rerio)* for creating a tool to unravel molecular mechanisms of toxicity, to discover novel biochemical biomarkers and to enable early diagnosis of effects.

The potential for ecotoxicoproteomics studies on the ecotoxicological model organism zebrafish was reviewed by several groups (Chen *et al.* 2004, Love *et al.* 2004; Miracle and Ankley 2005; Pichler *et al.* 2003, Scholz *et al.* 2008; Spitsbergen and Kent 2003). There are many advantages of using developmental stages of zebrafish in a proteomic approach. These include the sequenced genome allowing successful identification of proteins, the well known and easily observable lethal, and sublethal toxicity endpoints, the fast development, the easily available high number of organisms, no need for external feeding and the small size of the organisms. Hence, toxicity related responses can be studied in whole vertebrate organisms, but without performing ethically problematic animal experiments or using fish adults. But to date, only few proteomic approaches on developing zebrafish with toxicological background (Shrader *et al.* 2003) or also ontogenesis background (Link *et al.* 2006, Tay *et al.* 2006, Ziv *et al.* 2008) were realised. One of the major reasons is the high abundance of yolk proteins early in development of oviparous animals which could mask and reduce the sensitivity for the detection of changes in the cellular protein pattern. Ziv *et al.* (2008) have studied the proteome of zebrafish oocytes, when still not many yolk proteins have been inoculated in the eggs. Other studies have applied manual techniques to remove the yolk sacs in early embryos (Link *et al.* 2006, Tay *et al.* 2006) and thus, to reduce the yolk protein content. However, these techniques may interfere with the detection of stress-induced responses by the tested compounds.

Therefore, to fulfil the overall objective, the present thesis addresses several issues concerning the aim of developing a toxicoproteomics approach in developing zebrafish. These include:

(i) the creation of baseline proteomics data by proteome analysis of different developmental stages (from shortly after egg fertilisation until five days post fertilisation) during zebrafish development **(Chapter 3),**

(ii) the characterisation of toxic stress in embryos two days post fertilisation when still many yolk proteins are present **(Chapter 4)**,

(iii) the application of ecotoxicoproteomics with post-hatched embryonic stages containing less yolk proteins for addressing changes in the cellular protein pattern **(Chapter 5)**

(iv) and the deduction of novel biomarkers indicating toxic exposure or disturbed embryonal development from the applied ecotoxicoproteomics approaches **(Chapter 6)**.

The specific research goals and approaches addressing these four objectives are introduced, described and discussed in four separate consecutive chapters. All applied methods are presented in an overall methodology section.

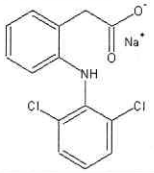
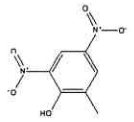
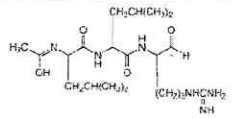
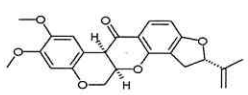
CHAPTER 2

Methodology

2.1 Physico-Chemical Properties of Characterised Toxicants

The following substances (Table 2-1) were chosen as reference toxicants for characterising the potential of toxicoproteomics approaches with developing zebrafish (Chapter 3-5) and to establish novel biomarkers (Chapter 6). Hereby, one generally embryotoxic compound (Ethanol) as well as substances with known and specific *Modes of Actions* [Diclofenac, Dinitro-ortho-cresol (DNOC), Leupeptin and Rotenone] were tested.

Table 2-1 Structure related data, industrial usage and biochemical targets are presented for the used toxicants

Toxicant	*Structure*	*MW (g/mol)*	*log K_{OW}*	*Henry constant [atm m^3/mol]*	*Industrial usage*	*Biochemical target*
Diclofenac CAS 15307-86-5		318.13	0.7	$4.73*10^{-12}$	Non-steroidal antiinflam-matory drug (NSAID)	Inhibition of Cyclo-oxygenases (COX 1 and COX 2)
4,6-Dinitro-ortho-cresol (DNOC) CAS 534-52-1		198.13	2.1	$1.4*10^{-6}$	Insecticide	Decoupler of oxidative phosphorylation
Ethanol CAS 64-17-5	$H_3C\text{-}CH_2OH$	46.06	-0.14	$5*10^{-6}$	Various appli-cations	Multiple targets
Leupeptin CAS 103476-89-7		427.56	0.29 a)	b)	Protease inhibitor	Inhibitor of Cathepsin L
Rotenone CAS 83-79-4		394.42	4.1	$1.4*10^{-13}$	Insecticide	Inhibition of electron transport chain (Complex I) in oxidative phosphorylation

a) Parameter was calculated based on structure formula with Cambridge Software ChemDraw and Chem Prop based on Crippen`s fragmentation method
b) No data or estimation available

DNOC and Rotenone were chosen because of their action in a primary energy metabolism pathway (oxidative phosphorylation/ATP-sythesis). In contrast, Diclofenac effects were studied since it affects a secondary biochemical pathway (inflammation). Leupeptin was tested because of its action as specific Cathepsin L inhibitor. The

background for selection of the chemicals is described and discussed in detail in the specific chapters.

2.2 *Fish Culture, Embryo Collection and Exposure Conditions*

Wild Type India Kalcutta (WIK) zebrafish were obtained from the Tübingen Zebrafish Stock Centre. Fish cultivation was carried out in glass aquaria (120 litres) with activated carbon filtered and equilibrated tap water at 27 ± 2 °C with a density of 5 fish per litre and a female to male ratio of 2:1 (Westerfield 2000). The light/dark regime was set to 12/12 h. The fish were fed twice daily with commercial dry food (Tetramin, Tetra, Melle, Germany) and once a day with *Artemia* sp. *nauplii* (Sanders, Odgen, UT, USA). The zebrafish are photoperiodic in their breeding and produce eggs in the morning, shortly after sunrise (Westerfield 2000). Therefore, mating and spawning were induced by the onset of light and by the addition of synthetic spawning substrates (plant imitations). Eggs were collected with a grid-covered dish and cleaned with aerated standard dilution water as specified in ISO 7346-3 (ISO-water). Fish eggs were controlled for fertilisation 1 h after spawning (in the four- to eight-cell stage) and unfertilised eggs were rejected. Fertilised eggs were incubated at 27.5 °C in ISO-water in glass dishes with a density of one egg per 2 ml. Developmental stages were classified according to Kimmel *et al.* (1995) using an inverse microscope (Olympus IX70-S8F, Hamburg, Germany) with a 50 x magnification.

2.2.1 Fish Embryo Test (DarT)

The fish embryo bioassay was performed according to the OECD Draft Guideline for Testing of Chemicals (OECD 2006). The fertilised eggs (four-to eight- cell stage) were exposed to toxicant dilutions two hours after spawning at the latest. The embryos were incubated each in 2 ml of the toxicant solution for 48 h at static conditions (temperature 27.5 °C, 12 h light/dark with 30 µmol photons $m^{-2} s^{-1}$). Toxicant dilutions were produced using aerated ISO-water. Nine to ten embryo replicates per toxicant concentration were examined and data were obtained from three independent experiments.

Depending on lipophilicity (log K_{OW}) of the toxicants, embryo bioassays were executed in 24 well polystyrene microtiterplates or in glass vials (Riedl *et al.* 2007, Schreiber *et al.* 2008).

Hydrophilic toxicants were assayed in polystyrene 24 well microplates (Greiner GmbH, Frickenhausen, Germany) or glass vials (see below) as recommended in OECD Draft Guideline (OECD 2006). One embryo was cultivated per well in 2 ml toxicant or control solution. Each plate comprised four internal control replicates (in ISO-water) and ten embryo replicates for two test concentrations each. Additionally, one external control plate with 24 control replicates was included in each experiment. Microplates were covered with a self-adhesive plastic film and a polysterol lid (Greiner GmbH, Frickenhausen; Germany) and were stored in translucent plastic bags.

The hydrophobic substance Rotenone was assayed in glass vials. If applied in glass vials, three embryos per vial in 6 ml solution were incubated. For each tested toxicant concentration, 3 vials (9 embryo replicates) and for one experiment at least 5 external controls were included. Glass vials were covered with a glass lid.

If toxicants were not well soluble in water (Rotenone, DNOC), they were solved in Dimethyl-sulfoxide (DMSO). The maximal concentration of DMSO in the test solutions was 0.01 %. In this case, 0.01 % DMSO was also added to the controls.

Only nominal concentrations of the chemicals are shown. Real concentrations of the chemicals during the assays have not been investigated. However, Riedl and Altenburger (2007) have described the microplate assay, as applied in this study, as effective for substances with a log K_{OW} lower than 3 and a Henry coefficient log K_{AW} higher than -4. Therefore, for Diclofenac and Leupeptin constant concentrations can be assumed during the 48 h exposure time. For Diclofenac, stable exposure concentration was demonstrated with the applied test regime and exposure time by a study in our group (Ulrike Krug, personal communication). The higher volatility of Ethanol and DNOC was addressed by using adhesive foils and an additional lid to cover the plates. Therefore, also for these toxicants stable exposure concentrations can be assumed. As recommended by Schreiber *et al.* (2008), the assay for Rotenone with a log K_{OW} of 4.1 was carried out in glass vials (as described above) to avoid concentration decrease during exposure.

Tested concentrations for the compounds were chosen individually for each tested compound in order to enable good modelling of concentration-response-relationships. All tested concentrations are summarised in Table 2-2.

After exposure, all embryos were analysed by inverse microscopy (Olympus IX70-S8F, Hamburg, Germany). Effect characterisation was carried out within one hour. At the end of the experiments embryos had an age of about 51 to 52 hours post fertilisation and are referred to as two days post fertilisation (2 dpf) old embryos. Coagulated eggs and

severe malformations such as absent blood circulation and absent heartbeat were considered as lethal toxicological endpoints. Sublethal toxic endpoints included effects like pigmentation impairments, retardation in development, affected heart beat and blood circulation, earlier hatching, distorted backbone, oedema in heart or yolk region. For toxicity evaluation, the observed lethal and/or sublethal effects were added up to the final percentage effect (within the ten replicates per concentration). Concentration-effect-relationships were established using a logistic model ($y = -100/ (1 + (x/x_0)\ ^p) + 100$, with x the concentration in % (v/v), x_0 the median effect concentration (EC50) and p the slope) with the ORIGIN software (Version Origin 7.5, Friedrichsdorf, Germany).

Table 2-2 Summary of all tested concentrations for determination of concentration-effect-relationships for all analysed compounds. Exposure time was 48 hours and started at 2 hpf (embryos) or 72 hpf (larvae) as indicated in the table.

Ethanol [mM] *2-50hpf*	***DNOC [µM]*** *2-50hpf*	***DNOC [µM]*** *79-127hpf*	***Diclofenac [µM]*** *2-50hpf*	***Diclofenac [µM]*** *79-127hpf*	***Rotenone [nM]*** *2-50hpf*	***Rotenone [nM]*** *79-127hpf*	***Leupeptin [mM]*** *2-50hpf*
5.14	0.06	0.050	0.0014	0.0014	0.0025	0.25	0.01
10.29	0.19	0.252	0.0029	0.0029	0.126	12.68	0.1
21.43	0.25	0.505	0.0059	0.0059	1.27	25.35	0.3
22.64	0.50	1.262	0.0115	0.0115	2.54	38.03	0.5
33.95	1.01	2.019	0.0229	0.0229	12.68	50.71	0.7
42.87	1.51	2.523	0.0459	0.0459	25.35	57.05	0.9
50.76	2.02	3.028	0.0918	0.0918	50.71	63.39	
85.74	2.52	3.533	0.1839	0.1839	126.77	69.73	
107.17	3.03	3.785			190.16	76.06	
114.89	3.53	4.038			253.55	101.42	
128.61	4.04	4.290			507.1	126.77	
171.48	4.54	4.542			1267.75	253.55	
214.35	5.05	4.795				1267.75	
257.22	5.55	5.047				2535.50	
342.96	15.14	7.570					
857.39	15.77	50.469					
1714.78	31.54						
	63.09						
	126.17						
	252.25						

2.2.2 Effect Determination in Larvae

The fish larvae bioassay applied in this study was carried out similar to the DarT and according to the OECD Draft Guidline for testing of Chemicals with the zebrafish embryo (OECD 2006) in terms of test regime, exposure time and toxic endpoints. It should be noticed that the fish early-life stage toxicity test (OECD 1992) which is actually available for chemical testing in developing fish until they are free feeding, starts

shortly after egg fertilisation and requires an exposure time of at least 5 days and was not applied. In this study in contrast, 79 hpf old hatched larvae were incubated according to DarT for 48 h in toxicant solutions with a density of one organism per 2 ml. The assay was carried out in 24 well microplates or glass vials, respectively under static conditions (temperature 27 ± 1 °C, 12 h light/dark with 30 mmol photons $m^{-2}s^{-1}$). The assay was restricted to *eleutheroembryos* which is the life interval between hatch (3 dpf) and the onset of exogenous feeding (5 dpf). Thus, no external feeding was supplied during the assays. All developmental stages between 3 dpf and 5 dpf are referred to as larval stages in this thesis.

After exposure all larvae were screened for effects by inverse microscopy (Olympus IX70-S8F, Hamburg, Germany). Coagulation, absent blood circulation and absent heartbeat were considered as lethal toxicological endpoints after 48 h exposure. Sublethal toxic endpoints included effects like oedema in the heart region, effects in cardiovascular functions and distorted backbone. For toxicity evaluation, the number of larvae with lethal and/or sublethal effects was added up to the final percentage effect (within the ten replicates per concentration) compared to the controls. Concentration-effect-relationships were established using a logistic model (y = 100 + (-100) / (1 + (x/x0)^p), with x as the concentration in % (v/v), x0 as the median effect concentration (EC50) and p as the slope) with the ORIGIN software (Version Origin 7.5, Friedrichsdorf, Germany).

2.2.3 Exposure Conditions for the Analysis of Toxic Responses at the Proteome Level

To characterise toxic effects at the molecular level of zebrafish embryos or larvae, individuals were collectively exposed. Twenty five (for analysis of the proteome of embryos) or fifty (for analysis of the proteome of larvae) individuals were exposed together to different concentrations of the toxicants in 200 ml glass vials with a density of one egg/ organism per 2 ml toxicant solution. For each condition three exposure replicates were included. Preceding exposure, fish eggs were sorted for fertilization (see DarT) and exposure started at least 2 to 3 hours post fertilisation for embryos and at 79 hpf for larvae. The glass vials were covered with glass lids and the fish eggs were incubated in the toxicant solutions for 48 h at static conditions (temperature 27.5 °C, 12 h light/dark with 30 µmol photons $m^{-2} s^{-1}$). After 48 h exposure time, embryos and larvae, respectively were analysed for morphological or physiological effects and except for

Ethanol (s. Chapter 4), non-affected organisms were pooled (20 organisms for one embryonal sample or 40 organisms for one larval sample) in 2 ml reaction tubes. The embryos were washed twice with 1 ml of *aqua dest.* and shock frozen in liquid nitrogen.

2.3 Protein Analysis

2.3.1 Protein Sample Preparation

Homogenisation of the frozen embryos or larvae was done in 10 µl lysis buffer [8 M Urea, 2 % CHAPS, 0.5 % v/v IPG-Buffer 3-10 linear (GE-Healthcare, Uppsala, Sweden), 1 % (v/v) protease inhibitor cocktail (SIGMA, Taufkirchen, Germany)] per organism (e.g. 200 µl for 20 embryos) using a metal-bladed homogenizer (Ultra Turrax, IKA Labortechnik, Staufen, Germany) for 30 s at a speed of 300 rpm.

The homogenates were centrifuged at 4 °C for 30 min at 9000 x g (SIGMA 2K15 Zentrifuge, Osterode, Germany). Total protein concentration in the supernatant was measured using the BIO-RAD DC Protein assay (BIO-RAD, Munich, Germany) in accordance to Lowry *et al.* (1995). Subsequently, protein samples were immediately used for 1D SDS PAGE or 2-DE analysis and not frozen again at -20 or -80 °C.

2.3.2 One Dimensional SDS-PAGE

SDS-PAGE was done according to Laemmli (1970). The embryo homogenates were diluted 1:2 with two-time SDS-PAGE Buffer (0.13 M Tis-HCl, 4.2 % (w/v) SDS, 21 % (v/v) Glycerin, 19 % (v/v) ß-Mercaptoethanol, Bromphenolblue), denaturated for 5 min at 95 °C and subsequently centrifuged for 15 min, at 9000 x g in a table centrifuge (5415 C Eppendorf, Hamburg, Germany). 30 µg protein was then loaded to a 3 % acrylamide/bisacrylamid collecting gel. For protein-separation, 12.5 % Acrylamide/Bisacrylamide separation gels were used (size: 10.1 x 8.3 cm) and separation was done at constant 150 V for 2 h at room temperature. Gels were stained by colloidal Coomassie staining with the ROTI BLUE® (Roth, Karlsruhe, Germany) staining solution, washed two times with 100 ml *aqua bidest.* and air-dried between two cellophane sheets (BioRad, München, Germany). For protein MW estimation, 5 µl of protein standard marker (peqGOLD Protein-Marker I, Peqlab®, Erlangen, Germany) was run alongside of each gel.

2.3.3 Two-Dimensional Gelelectrophoresis (2-DE)

The two-dimensional electrophoresis was carried out according to Görg *et al.* (1987) with immobilized pH gradients-IEF in the first, and SDS-PAGE in the second dimension.

2-DE Analysis of the Proteome of Zebrafish at Different Developmental Stages and Toxicoproteomics Analysis with 2 dpf old Embryos

The following methods were used to obtain the results described in Chapters 3 and 4. Preceding the isoelectric focussing (IEF), the protein samples were diluted 1:4 with dilution buffer [20 mM DTT, 8 M urea, 2 % CHAPS, 0.5 % v/v IPG-Buffer 3-10 linear (GE-Healthcare, Uppsala, Sweden)]. The IEF was performed using the Ettan IPGphor (GE Healthcare, Uppsala, Sweden) and 18 cm linear, pH 3-10 Immobiline Dry Strips (GE Healthcare, Uppsala, Sweden). Rehydration was carried out at room temperature overnight with 400 µl rehydration solution per Dry Strip [DeStreak solution (GE-Healthcare, Uppsala, Sweden), 0.5 % v/v IPG-Buffer 3-10 linear].
50 µg of protein samples were subjected to IEF via cup loading (anode) and the focussing was performed under following conditions: 150 V, 1.5 h; 300 V, 2 h; 600 V, 2 h; 8000 V gradient, 0.5 h; 8000 V constant, 7 h, all steps at 20 °C. After IEF, the Immobiline Dry Strips were equilibrated at room temperature for 15 min in a buffer containing 50 mM Tris-HCl, 6 M Urea, 30 % v/v Glycerol, 2 % w/v SDS, 0.002 % w/v Bromophenol blue and 2 % w/v DTT, and subsequently alkylated for 15 min in a buffer containing 50 mM Tris-HCl, 6 M Urea, 30 % v/v Glycerol, 2 % w/v SDS, 0.002 % w/v Bromophenol blue and 4.5 % w/v Iodacetamid.

For the SDS-PAGE, as the second-dimension, the IPG strips were sealed on the top of 1 mm thick 12.5 % or 13 % Polyacrylamide gels (27.6 x 21.7 cm). Vertical electrophoresis was carried out overnight (16 h) at 12 °C and about 1 W/gel. Gels were silver stained according to Heukeshoven and Dernick (1988) and Yan *et al.* (2000) and digitalised at a resolution of 200 dpi using the image scanner (GE-Healthcare, Uppsala, Sweden). Subsequently, gels were air-dried between two cellophane sheets (Biorad, Munich, Germany) at room temperature.

2-DE Conditions for Toxicoproteomics Analysis in 5 dpf Old Zebrafish Larvae

The following methods were used to obtain the results described in Chapters 5. The 2-DE was carried out as described above but for IEF 18 cm linear immobiline dry strips with pH range of pH 4-7 were used instead. Moreover, sample loading was done via cup-loading at the cathode. 14 % Polyacrylamide gels were used for the second dimension. All other 2-DE conditions and staining, digitalisation and washing of the gels were performed as described before.

2.3.4 Image Analysis and Statistics

Densitometric image analysis for the 2-DE experiments was performed using the software package Delta 2D (Version 3.4, DECODON, Greifswald, Germany). To enable the same protein pattern on each gel (100 % matching strategy, for spot detection, a fusion gel, including all gels of the experiment, was created after gel matching. All proteins were detected and edited on this gel. Subsequently, the detected spot pattern was transferred to all gels in the experiment. The amount of protein present in a spot was described as the area of the spot multiplied by the pixel density and referred to as the volume. Individual spot volumes were normalised to the total protein amount detected within each gel and the amount of each spot therefore was expressed as a relative volume. All analysed conditions were run at least in triplicates. Due to the inherent semi-quantitative silver staining method the 10 % largest protein spots were excluded from the normalisation set.

Statistical Analysis of the Proteome Data

Two different analysis methods were applied for comparison of control gels and gels derived from toxicant exposure: a multivariate pattern analysis method to compare the whole proteome pattern and a univariate spot-to-spot analysis method to obtain detailed information on single spots that have changed in expression after toxicant exposure.

Principle component analysis (PCA) method was applied to compare protein patterns. All detected proteins in one experiments were included in the multivariate analysis methods. PCAs were carried out using the software package Jump (Version 7.0, Cary, USA)

Proteins found to be different in control and exposure events were analysed for significance using a Student's *t*-test. Only spots showing a significant ($p < 0.05$) and at least two-fold (for silver staining) difference in abundance were considered as up- or down-regulated proteins. Here the term up-regulation (or over-expression) refers to elevated amounts of proteins on the gels and implies both new synthesis of proteins as well as fragments derived from already existing proteins by proteolytic processes.

2.3.5 Identification of Proteins

Proteins of interest were excised from the stained gels. Following silver purification, the spots were subjected to in-gel trypsin digestion as previously described in Benndorf *et al.* (1997). The resulting peptides were separated by RP nano-LC (LC1100 series, Agilent Technologies, Palo Alto, California; column: Zorbax 300SB-C18, 3.5 mm, 15060.075 mm; eluate: 0.1 % formic acid, 0-60 % Acetonitrile) and analysed by MS/MS (LC/MSD TRAP XCT mass spectrometer, Agilent Technologies). MS data were submitted to the online MASCOT (http://www.matrixscience.com) and searched against the nrNCBI (National Centre for Biotechnology Information) protein database. The identified proteins were characterised via the Swiss Prot and TrEMBL (www.expasy.org/sprot) databases. All estimated molecular weights were calculated by the online PROTPARAM tool (www.expasy.org/tools/protparam.html).

2.3.6 Immunoblot Analysis

The 1D SDS-PAGE was carried out on 12.5 % polyacrylamide gels using the method of Laemmli (1970) as described above. 2-DE for immunoblot analysis was performed using 7 cm linear, pH 3-10 Immobiline DryStrips (GE-Healthcare, Uppsala, Sweden). 7 µg of the protein samples were loaded on the strips via cup-loading. IEF was done with the following IEF-program (150 V, 1.5 h; 300 V, 2 h; 600 V, 1 h; 5000 V gradient, 0.5 h; 5000 V constant, 2.5 h). After equilibration and alkylation (performed as described before), the strips were sealed on the top of 12.5 % acrylamide/ bisacrylamide gels (size: 10.1 x 8.3 cm) and separation was done at constant 150 V for 2 h at room temperature. Subsequently, the gels were electroblotted onto nitrocellulose membranes for 2 h at 150 V. Following overnight blocking (20 mM Tris, 150 mM NaCl, 3 % w/v Gelatin, pH 7.5) and washing (20 mM Tris, 150 mM NaCl, 0.05 % v/v Tween-20, pH 7.5) of the membranes, the primary antibody, a polyclonal rabbit anti-zebrafish vitellogenin antibody (DR-264, Biosense Laboratories, Bergen, Norway), was incubated

for 2 h. The membranes then were washed with the above washing buffer and treated with the secondary goat anti rabbit IgG antibody (SIGMA-ALDRICH, Taufkichen, Germany) coupled with horseradish peroxidase. After washing, the immunoblot assays were visualised using a chemoluminescence kit (Pierce, Rockford, USA) with the FluoroChem 8900 (alpha Innotech, San Leandro, USA) imaging system.

2.4 Cathepsin Enzymatic Activity Analysis

The following methods were used to obtain the results described in Chapter 6.

2.4.1 *Danio rerio* Embryo Collection for Enzymatic Measurements at Different Developmental Stages

For characterisation of cathepsin activities at different DR developmental stages, embryos at 0.5, 2.5, 9, 27, 49, 73, 97 and 126 hpf were collected in 2 ml Eppendorf® reaction tubes, washed two times with 1 ml of *aqua dest.* and immediately frozen in liquid nitrogen. For each developmental stage 30 intact embryos were pooled in one reaction tube and referred to as one replicate. For each developmental stage at least three replicates were analysed.

2.4.2 Exposure Conditions to Study *in vivo* Effects of Leupeptin, DNOC, Ethanol and Rotenone on Cathepsin Activities

For analysis of cathepsin activities under toxic exposure 70 to 90 embryos were collectively incubated for 48 h in the test solutions (Leupeptin, DNOC, Ethanol, Rotenone) or ISO-water (controls) in 200 ml glass vials. Tested concentrations were chosen according to the experiments and are described in detail in Chapter 6. After exposure, all embryos were screened for morphological effects and 60 embryos that were intact by visual inspection, were pooled in one reaction tube and referred to as one replicate. These were washed at least 6 times with 1 ml of *aqua dest.* before freezing in liquid nitrogen and storage at -80 °C. For each exposure condition at least three independent replicates from the same spawning event were performed. Moreover, the exposure experiment was repeated three times at different days and from different experimentators. Hence, nine under same condition exposed replicates, including 60 embryos each, were used for the cathepsins studies. For analysis of concentration

dependence on cathepsin activities, one replicate with 60 embryos for each concentration was included in the experiment.

2.4.3 Protein Isolation from *Danio rerio* Embryos

For *in vivo* enzymatic assays, *Danio rerio* embryos were homogenised for 30 s on ice in Lysis Buffer (0.1 M sodium acetate, 1 mM EDTA, 0.1 % Triton x-100, pH 4) (Dolenc *et al.* 1992) with a concentration of 10 µl buffer per DR embryo using a metal-bladed homogeniser (Ultra Turrax, IKA Labortechnik, Staufen, Germany). The homogenates were then centrifuged at 4 °C for 30 min at 9000 x g (Sigma 2K15 centrifuge, Osterode, Germany) and the supernatant was stored on ice for max. 30 min before analysis of the sample. The protein concentration was measured according to Lowry *et al.* (1951) using the BioRad DC protein assay (BioRad, Munich, Germany).

2.4.4 Cathepsins Assays (CatC, CatH, CatL)

The cathepsin assays were carried out in black 96-well microplates (Schubert-Laborbedarf, Leipzig, Germany). All assays are based on the conversion of cathepsin specific substrates to the fluorogenic product 7-Amino-4-methylcoumarin (AMC). Fluorescence was measured 60 min after start of the assay reaction in a microplate spectrofluorometer (SpectraMax Germini, Molecular Devices) at 360 nm excitation and 460 nm emission. According to InnoZyme™ Cathepsin L activity kit (Calbiochem, Gibbstown, USA) all assays were carried out in 200 µl assay solution in four steps: At first, 50 µl of assay buffer (0.4 M acetic Acid, 4 mM Na EDTA, pH 5.5) and secondly, 50 µl of either DR protein sample, lysis buffer (see above) (blanks), AMC standard or pure cathepsins were added into the wells. The plates were sealed with a self-adhesive plastic film and gently shaken for 15 min at room temperature. In a third step, 50 µl of diluent [0.1 % Brij® 35 detergent (Polyoxyethyleneglycol dodecyl ether), Calbiochem, for blanks and total substrate conversion] or inhibitor (for unspecific substrate conversion) diluted with Brij were added. The plate was gently shaken again for 15 min at room temperature. The reaction was started by adding 50 µl of substrate solutions (0.1 mM final concentration for all assays). Plates were then incubated for 60 min at 37 °C.

To obtain information about unspecific substrate conversion for cathepsins L and H, each sample was measured twice, with and without addition of a specific CatL/H

inhibitor (third step above). Activity for specific substrate conversion was then obtained by subtracting both values according to equation 1 (Eq. 1) after blank normalisation.

$$S_S = S_T - S_U \qquad \text{Eq. 1}$$

(with S_S specific substrate conversion, S_T total substrate conversion = substrate conversion without inhibitor, S_U unspecific substrate conversion = substrate conversion after addition of inhibitor)

Specificity of the cathepsin substrate conversion in the applied enzymatic assays were analysed because cathepsins belong to a conserved and homologous enzyme family (Fabra and Cerda 2004) and unspecific substrate conversion processes can be well expected. All measured specific as well as unspecific enzymatic reactions could contribute to yolk processing (vitellogenin degradation) and hence were included in the considerations (Chapter 6). Stable unspecific substrate conversion was detected for CatL and CatH substrates. These could be either due to homologous cathepsins in the samples or also by presence of other proteases like aminopeptidase B, cleaving unspecifically CatH substrate (Barrett 1980). CatC substrate (H-Gly-Arg-AMC) conversion is highly specific for Dipeptidylaminopeptidase I (CatC) (Chan *et al.* 1985) and hence no unspecific reaction was detected and assumed for CatC.

Specificities of substrate conversions of all used substrates and inhibitor specificities for all used inhibitors were controlled with pure cathepsins L, H (Calbiochem, Gibbstown, USA) and C (Type X, Sigma-Aldrich, Taufkirchen, Germany).

Cathepsin L. Cat L activity was assayed against the Z-Phe-Arg-AMC substrate (Calbiochem, Gibbstown, USA). To avoid interference with cathepsin B the CatB inhibitor CA-074 (1 µM final concentration, Calbiochem, Gibbstown, USA) was added to the activation buffer. As specific CatL inhibitor Z-Phe-Tyr-(tBu)-Diazomethylketone (10 µM final concentration, Alexis Biochemicals, Lörrach, Germany) was used. 10 µM final CatL inhibitor concentration is recommended in the InnoZyme™ Cathepsin L activity kit (Calbiochem, Gibbstown, USA). Results of this study have shown that 10 µM final CatL inhibitor can inhibit 92 % activity of 1700 mU CatL (data not shown) which was about the highest detected CatL substrate conversion activity in the embryo samples (see Chapter 6, 6.2.1, Figure 6-2).

Cathepsin H. CatH activity was assayed against the substrate H-Arg-AMC (Bachem, Weil, Germany). As a specific CatH inhibitor H-Leu-chloromethylketone

(100 µM final concentration, Bachem Weil, Germany) was used. It was determined that 10 µM final concentration of CatH inhibitor is enough to completely inhibit 23 mU CatH in the applied assay conditions (data not shown). The maximal measured CatH activity was 200 mU (see Chapter 6, 6.2.1, Figure 6-2). It was concluded that 100 µM of CatH inhibitor (as applied in this study) should be enough to totally inhibit CatH in the samples.

Cathepsin C. For CatC activity determination G-Gly-Arg-AMC (Bachem, Weil, Germany) was used as a specific substrate. No unspecific substrate conversion was observed with the tested pure cathepsins assayed in this study. This is in accordance with literature data describing G-Gly-Arg-AMC as very specific substrate for CatC (Chan *et al.* 1985).

All activities were calculated as U (1 µMol substrate/min). For obtaining specific activities, activity data were related to protein content in the sample (U/mg total protein). The protein concentration in the samples was determined according to Lowry *et al.* (1951) using the BioRad DC protein assay (BioRad, Munich, Germany). It should be noticed that in the present work the term of specific substrate conversion is related to the specificity of the enzyme and is different from the term of specific activity which is defined as the activity of the enzyme related to the total protein amount in the sample.

Statistical analysis to test for significant differences in cathepsins activities between controls and exposure conditions were performed with SPSS (Version14.0 for Windows, USA) and using the Mann Whitney U-Test.

CHAPTER 3

Proteome Analysis during the Development of Zebrafish

3.1 Introduction

In contrast to adult animals, proteome analysis of developing organisms addresses a biological system which is highly variable in terms of physiological, morphological, etc. parameters. It can be well expected that this variability is mirrored at the molecular level and may strongly influence all toxicoproteomics applications with zebrafish in developmental stages. Only one study has investigated the changes in the proteome of zebrafish during development (Tay *et al.* 2006) but by using deyolked organisms. Deyolking was proposed by Link *et al.* (2006) who have found that the proteome early in zebrafish development is dominated by yolk proteins which could hamper the analysis of cellular proteins. However, deyolking means the manual removal of the yolk which could interfere with toxicity based analyses and should be avoided in ecotoxicological applications. Therefore, this chapter addresses the investigation of changes in the proteomes from different developmental stages of whole non-deyolked zebrafish.

Only one proteomics study on zebrafish included yolk proteins in the proteomic analysis by not deyolking the embryos (Shrader et al. 2003). In oviparous vertebrates such as fish, yolk proteins are critical for regular embryonic development providing a variety of nutrients (Wallace and Jared 1969). Besides minor amounts of vitamin-carrying proteins, riboflavin- and thiamine- binding proteins, albumin, transferrin, immunoglobulin G, and α2-glycoprotein, two classes of phosphoglycoproteins, phosvitins and lipovitellins are the major proteins found in the yolk (Wallace and Jared 1969). These two phosphoglycoproteins derive from common precursor proteins called vitellogenins (Vtgs). Vtgs are encoded by a multigene family and constitute the major yolk proteins occurring in all oviparous animals (Byrne et al. 1989). They provide the embryo with essential nutrients including amino acids, lipids, metal ions, phosphates and carbohydrates. Therefore Vtg synthesis and degradation processes are important for regular oogenesis and embryogenesis and have been addressed in several studies (Byrne *et al.* 1989, Fagotto 1995, Heesen and Engels 1973, Korfsmeier 1966, Tyler and Sumpter 1996, Wallace 1985, Wallace and Jared 1969). The zebrafish Vtgs are expressed from seven different *Vtgs* genes and are classified in three types based on their primary amino acid sequence: Type I includes Vtg1 and Vtg4-7, Type II contains Vtg2 and Type III includes Vtg3. Due to its high expression (100 x higher than Vtg2 and 1000x higher than Vtg3) Type I Vtg is the predominant form of Vtg in zebrafish (Wang *et al.* 2005) (Figure 3-1).

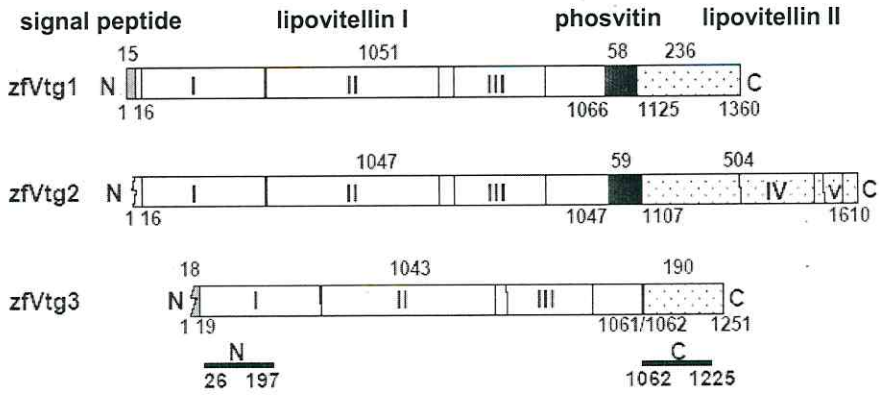


Figure 3-1 Schematical representation of the three distinct types of zebrafish Vtg (zfVtg1-3) proteins. Signal peptide, lipovitellin I, phosvitin, and lipovitellin II are marked by grey, white, black, and dotted boxes, respectively. The length of each domain (number of amino acid residues) and the positions of their boundaries are shown above and below the boxes, respectively. Homologous subdomains I–V are indicated inside the boxes. Two regions labelled by "N" and "C" are used for phylogenetic analyses and the amino acid numbers are based on zfVtg3 sequence. The figure was modified after Wang *et al.* (2005).

Vtg synthesis is tissue-, stage-and sex-dependent and under hormonal regulation. Vtg mRNAs are predominantly expressed in the female liver under the control of estrogen or 17β-estradiol (Wallace 1985, Wang *et al.* 2005). Following synthesis in the endoplasmatic reticulum, Vtgs undergo substantial modifications like glycosylation and phosphorylation, are then loaded with lipids and are subsequently secreted into the bloodstream. Upon ovarian uptake via specialised receptor-mediated endocytosis, Vtgs are proteolytically cleaved yielding the heavy (approx. 120 kDa) and light (approx. 30-35 kDa) chain lipovitellins (Lv) and phosvitins (Pv) (approx. 6 kDa) (Byrne *et al.* 1989). Phosvitins consist of greater than 50 % phosphorylated serine residues and therefore serve as rich sources of phosphate and metal ions such as calcium and iron. They may be required for embryonic bone formation (Taborsky 1980). Lipovitellins are the major lipid-proteins in the yolk and can be loaded with non-covalently bound lipids up to about 16 % w/w lipid (Montorzi *et al.* 1995, Raag *et al.* 1988). In addition, Lv interacts with Zn^{2+} ions and thus may serve as metal ion source (Montorzi *et al. 1995).* The exact mode of utilisation of the major yolk proteins and their timed release during embryogenesis has not been clearly established. However, it is widely accepted that the proteolytic cleavage processes of lipovitellins and phosvitins are directed and critical for normal embryonic development (Kanaya *et al.* 2000, Tay *et al.* 2006).

The objective of the study described in this chapter was the establishment of baseline proteomic data in zebrafish at different developmental stages, from shortly after

egg fertilisation until free feeding larvae. Yolk utilisation processes and the changing ratio between yolk- and cellular proteins during the development are an important issues being addressed in this chapter.

All tested developmental stages were embryonal stages. Organisms until 2 dpf were termed as embryos and all organisms between hatching (3 dpf) and the onset of external feeding (5dpf), eleutheroembryos, were referred to as larvae.

3.2 Results

3.2.1 Characterisation of the Protein Composition of Zebrafish Embryos at Different Developmental Stages

In a first step towards a proteomic approach for assessing ecotoxicological effects using zebrafish in early life stages we established reference protein patterns at different developmental stages (Figure 3-2). The 2-DE gels from eight distinct embryonic developmental stages, classified according to Kimmel *et al.* (1995), are depicted. In order to get an overview of the embryonic protein composition, the protein separation was done with a wide pH- range (pH 3 to 10) and limits of molecular weight variations between 250 kDa and 6.5 kDa. The fish egg samples for each developmental stage were independently obtained from different spawning events.

Development of zebrafish embryos within the chorion is initiated 45 min after fertilisation by the cleavage period in which the 1-cell stage develops to the 64-cell stage. Two hours post fertilisation the blastula period starts where cells are further divided before cell differentiation is initiated. Figure 3-2 A shows the 256-cell stage from the blastula period after 2.5 hpf where the cells are still undifferentiated. This was therefore chosen as the first developmental stage to be analysed by 2-DE. In Fig. 3-2B the 90 % - epiboly stage at 9 hpf is depicted which belongs to the gastrula period directly following the blastula stages. The subsequent segmentation period is featured by somite- and eye-formation and is followed by the pharyngula period from which two stages the Primordium-5 stage (Figure 3-2 C) at 26 hpf and the Primordium-15 stage (Figure 3-2 D) at 32 hpf are shown. Besides appearance of early pigmentation in retina and skin, spontaneous movements also appear at 26 hpf. The 32 hpf stage is characterised by detectable heartbeat which leads to strong blood circulation at 36 hpf. Figure 3-2 E and 3-2F show developmental stages from the hatching period, the long-pectoral stage at 48 hpf (Figure 3-2 E), and the protruding-mouth stage at 72 hpf (Figure 3-2 F). At 72 hpf

hatching of the embryos occurs. Figure 2-2 G and 2-2 H show zebrafish in the early larval period, after 4 days post fertilisation (dpf) and 5 dpf. Five day old larvae have a completed mouth system and could start with exogenous feeding. They show a strongly reduced yolk.

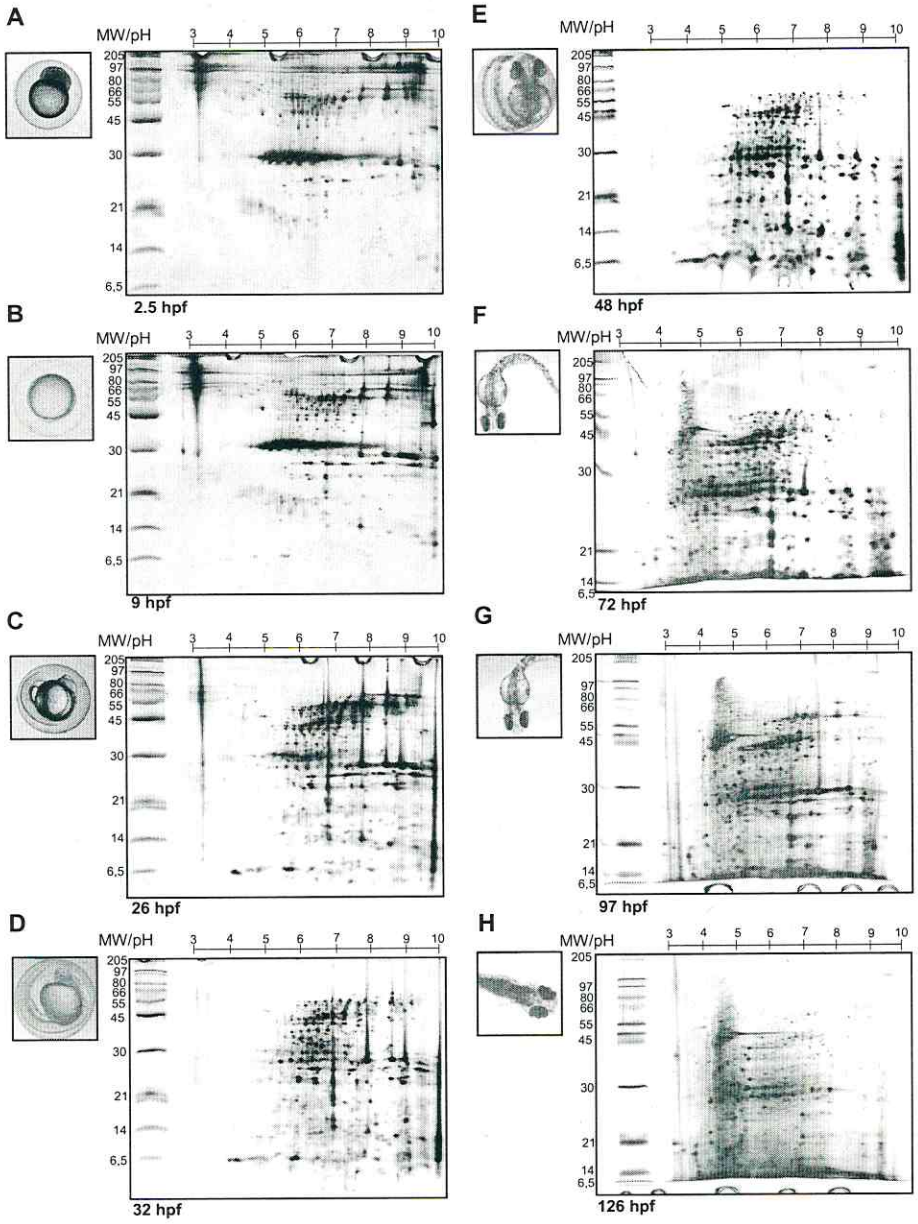


Figure 3-2 Zebrafish embryos at eight different developmental stages [(**A**) 2.5, (**B**) 9, (**C**) 26, (**D**) 32, (**E**) 48, (**F**) 72, (**G**) 97 and (**H**) 126 hpf] and their corresponding 2-DE protein expression pattern are depicted. For MW estimation MW standards were run alongside the embryonic protein samples.

Characteristic protein patterns could be detected for all embryonic and larval developmental stages shown. However, four types of protein patterns could be discriminated showing similar protein distribution over the gel. These are referred to as Type I, Type II, Type III and Type IV protein patterns. The first type of protein patterns, including 2.5 and 9 hpf old embryos (Figures 3-2 A and 2-2 B), is characterised by a high amount of high molecular weight proteins and two characteristic protein clusters: one acidic protein cluster (Cluster 1) around 80 to 97 kDa and pH 3-3.3 and one protein cluster of proteins (Cluster 2) having molecular weights around 30 kDa and pI's ranging from 4.8 to 7. There were no dramatically changes within the protein patterns from 2.5 hpf to 9 hpf but slight variances in the spot numbers on the 2-DE gels: 2.5 hpf embryos showed about 420 spots whereas around 500 protein spots were seen at 9 hpf. New proteins mainly occurred in gel regions between 45- 65 kDa and pH 5-7, but some also had lower MWs between 6 and 21 kDa. The constant Type I protein pattern within the first 9 hours of development are confirmed by 1D SDS-PAGE results (Figure 3-3). As embryos at the blastula and gastrula stages mainly consist of yolk and only a few embryonic cells the protein pattern on the gels may reflect mostly the yolk protein composition.

Type II protein patterns include protein patterns deriving from 26, 32 and 48 hpf embryos (Figures 3-2 C – 3-2 E). The two protein complexes seen in 2.5 and 9 hpf old embryos were strongly reduced. Many new proteins, having mainly lower molecular weights, were observed. With around 700 detectable proteins, there is an extensive increase in number of spots compared with the Type I protein pattern. The Type II patterns differ slightly in their protein composition below 30 kDa and in the number of detectable proteins. The 1D SDS-PAGE (Figure 3-3) shows a decrease in the number of proteins bands from 26 to 48 hpf.

The 2-DE gels derived from hatched 72 hpf old embryos and 97 hpf old larvae are characterised by many newly-appearing proteins with acidic pIs and hence are referred to as the Type III gels protein pattern.

In comparison to younger stages, a clear shift of the proteome towards proteins with acidic pIs was seen in 5 dpf old larvae and only few proteins which have been detected in younger embryos were obtained. As five day old larvae do not show a yolk anymore, the observed proteome seems to contain only less yolk- but more cellular-proteins and was hence considered as Type IV protein pattern. The differences in the

protein samples of 5 dpf old larvae compared to younger stages can also be followed by separating the proteins only according to their molecular weight (Figure 3-3).

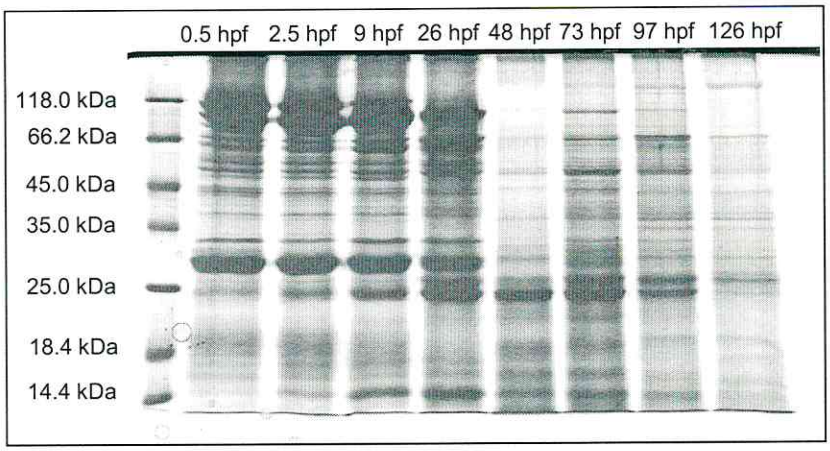


Figure 3-3 A coomassie stained 1-D 12.5 % SDS-PAGE showing protein compositions of zebrafish embryos at eight different time points [from shortly after fertilisation (0.5 hpf) to free feeding larvae (126 hpf)] during embryonal development is shown. 30 µg protein was loaded on each lane.

In summary, the results for the first time show developmental stage specific protein patterns of zebrafish during development. The protein patterns of whole zebrafish embryos therefore seem to be stable for the first 10 hpf of embryonic development (Type I gels: gastrula and blastula stage) before they dramatically change within the next 10 hours in the segmentation period. They subsequently remain constant again until the hatching and early larval period.

3.2.2 Identification of Proteins in 2 dpf old Embryos

For further characterisation of the protein profiles of whole zebrafish embryos and to get an impression about the protein identification success of the applied method, proteins from 2-DE gels of 2 dpf old embryos were excised and identified via an ESI-MS/MS approach. All identified spots are shown in Figure 3-4. The Mascot scores, accession numbers, MWs and pIs of all identified proteins are summarised in Table 3-1.

The applied MS compatible silver staining method (Yan *et al.* 2000) allow the identification of most of the excised proteins. Hereby, high validation of identification results could be obtained by MASCOT scores having values up to 900 (oe80). Most of the analysed proteins (93 %) could clearly be assigned to fragments of the yolk vitellogenins (Vtgs), except three remaining proteins which were identified as actin (muscular filamental protein), enolase (glycolysis enzyme) and histone H2A. 90 % of all

identified Vtg derivatives matched Vtg1. Additionally, four other Type I Vtg proteins including cleavage products from Vtg4 (c16) and Vtg7 (oe41, c11, oe69) were detected. Moreover, three identified proteins matched with Vtg2 (oe39, oe38, oe86) (Type II Vtg) which is expressed 100 times less then Vtg1 (Wang *et al.* 2005).

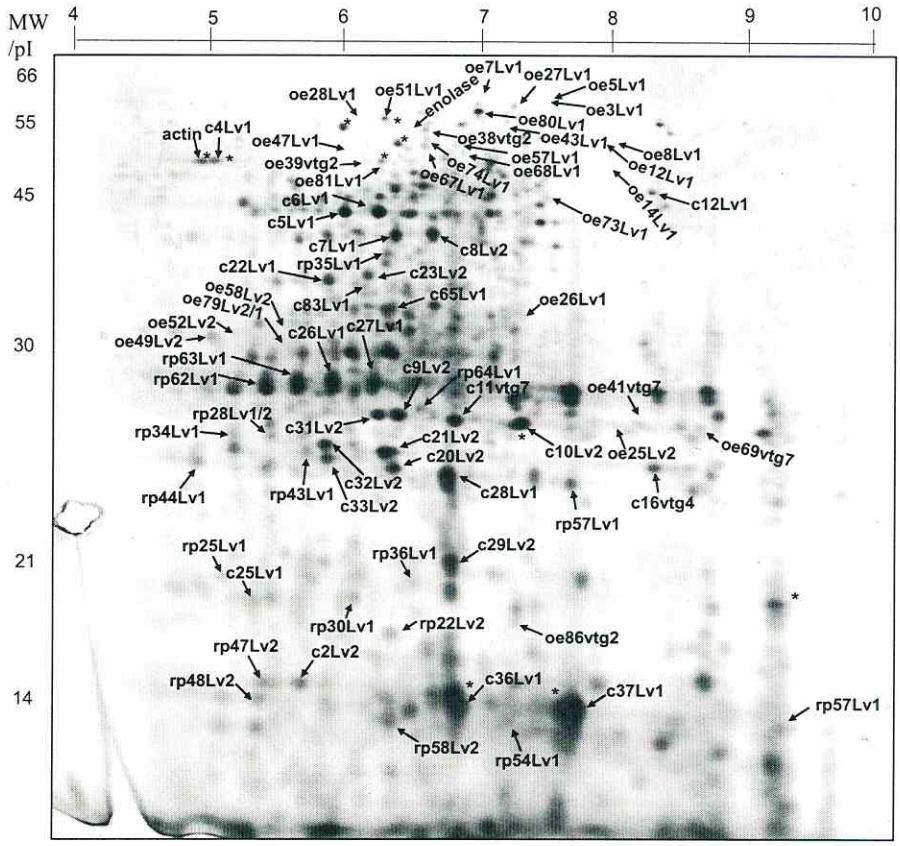


Figure 3-4 Location of all identified proteins in a 2-DE gel from protein samples of control 2 dpf old zebrafish embryos. Most of the identified proteins are derivatives from Vitellogenin 1 (Vtg1) and are termed as Lipovitellins (Lv), Lv1 being the N-terminal part and Lv2 being the C-terminal part of Vtgs. All proteins different from vitellogenins are nominated on the gel. Spots for identification were chosen according to results of an Ethanol exposure experiment (described in Chapter 4), but as they appear with different abundances in the proteome of control 2 dpf old zebrafish embryos, they are all depicted at the present gel [27 control spots (prefix c), 47 spots changed in abundance after Ethanol exposure (prefix oe-overexpressed, prefix rp-repressed)].

It is well known that upon ovarian uptake vitellogenins are cleaved into three domains including the N- and C-terminal lipovitellins-domains (Lv1 and Lv2) and the phosvitin (5.8 kDa)-domain as indicated in Figure 3-1 (Byrne *et al.* 1989, Montorzi *et al.* 1995, Patino and Sullivan 2002, Wallace 1985). Whereas no phosvitin was detected, all identified Vtg fragments matched to the N-terminal Lv1 (116.7 kDa) and C-terminal Lv2 (26.7-56.9 kDa), respectively.

Table 3-1 The label, protein name, MASCOT scores and accession numbers of all identified proteins are presented. Moreover observed and theoretical (calculated) molecular weight (kDa) and pI values are shown. Eight spots were identified with only low MASCOT scores (marked by asterisks), below the significance level of 50. But as they matched *Danio rerio* Vtg sequences after database search against all entries they are also shown in the table.

Spot NAME (ID)	*Protein-NAME*	*MASCOT score*	*Accession Numbers*	*MW (kDa) obs/calc[a]*	*pI obs/calc[a]*
c4	alpha cardiac actin	377	AAF20165	50/42.3	5.2/5.29
oe40	Enolase-3 (fragment)	138	Q3B7R7	53/47.4	6.5/6.19
c31	sim. to histone H2A	69	XP688374	27/25	6.4/11.22
oe80	Vitellogenin1	900	Q1LWN2	57	7.0
oe79	Vitellogenin1	569	Q1LWN2	30	5.6
oe5	Vitellogenin1	86	Q1LWN2	59	7.5
oe27	Vitellogenin1	610	Q1LWN2	58	7.5
oe7	Vitellogenin1	205	Q1LWN2	60	7.0
oe73	Vitellogenin1	195	Q1LWN2	45	7.5
oe51	Vitellogenin1	244	Q1LWN2	56	6.3
oe58	Vitellogenin1	277	Q1LWN2	32	5.6
oe52	Vitellogenin1	42*	Q1LWN2	31	5.3
oe12	Vitellogenin1	243	Q1LWN2	55	7.9
oe3	Vitellogenin1	641	Q1LWN2	58	7.5
oe67	Vitellogenin1	433	Q1LWN2	50	6.6
oe57	Vitellogenin1	274	Q1LWN2	53	6.9
oe47	Vitellogenin1	59	Q1LWN2	52	6.1
oe43	Vitellogenin1	595	Q1LWN2	56	7.3
oe28	Vitellogenin1	244	Q1LWN2	56	6.2
oe26	Vitellogenin1	91	Q1LWN2	33	7.4
oe14	Vitellogenin1	366	Q1LWN2	49	8.0
oe8	Vitellogenin1	196	Q1LWN2	55	8.0
oe81	Vitellogenin1	273	Q1LWN2	49	6.3
oe68	Vitellogenin1	171	Q1LWN2	51	6.9
oe74	Vitellogenin1	191	Q1LWN2	53	6.6
c2	Vitellogenin1	356	Q1LWN2	16	5.7
c4	Vitellogenin1	188	Q1LWN2	50	5.1
c5	Vitellogenin1	256	Q1LWN2	43	6.0
c6	Vitellogenin1	108	Q1LWN2	43	6.3
c7	Vitellogenin1	192	Q1LWN2	38	6.4
c25	Vitellogenin1	73	Q1LWN2	17	5.4
c8	Vitellogenin1	260	Q1LWN2	38	6.6
c9	Vitellogenin1	237	Q1LWN2	27	6.5
c10	Vitellogenin1	255	Q1LWN2	27	7.4
c12	Vitellogenin1	53	Q1LWN2	47	8.3
c20	Vitellogenin1	217	Q1LWN2	24	6.5
c23	Vitellogenin1	41*	Q1LWN2	35	6.3
c21	Vitellogenin1	232	Q1LWN2	25	6.4
c36	Vitellogenin1	47*	Q1LWN2	14	6.9
c33	Vitellogenin1	244	Q1LWN2	25	6.0
c32	Vitellogenin1	259	Q1LWN2	26	6.0
c31	Vitellogenin1	239	Q1LWN2	27	6.4
c29	Vitellogenin1	85	Q1LWN2	21	6.9
c28	Vitellogenin1	39*	Q1LWN2	24	6.9
c26	Vitellogenin1	192	Q1LWN2	28	6.0
c27	Vitellogenin1	118	Q1LWN2	28	6.3
c22	Vitellogenin1	76	Q1LWN2	34	5.9
c37	Vitellogenin1	127	Q1LWN2	14	7.9

c83	Vitellogenin1	260	Q1LWN2	34	6.2
c65	Vitellogenin1	268	Q1LWN2	33	6.3
rp22	Vitellogenin1	107	Q1LWN2	18	6.4
rp28	Vitellogenin1	135	Q1LWN2	26	5.5
rp63	Vitellogenin1	387	Q1LWN2	28	5.7
rp64	Vitellogenin1	92	Q1LWN2	27	6.6
rp58	Vitellogenin1	73	Q1LWN2	13	6.4
rp57	Vitellogenin1	47*	Q1LWN2	13	9.2
rp54	Vitellogenin1	31*	Q1LWN2	13	7.3
rp48	Vitellogenin1	107	Q1LWN2	15	5.3
rp47	Vitellogenin1	156	Q1LWN2	16	5.4
rp44	Vitellogenin1	96	Q1LWN2	25	4.9
rp43	Vitellogenin1	76	Q1LWN2	24	5.8
rp36	Vitellogenin1	90	Q1LWN2	20	6.5
rp34	Vitellogenin1	121	Q1LWN2	26	5.5
rp30	Vitellogenin1	116	Q1LWN2	19	6.1
rp25	Vitellogenin1	32*	Q1LWN2	21	5.2
rp62	Vitellogenin1	432	Q1LWN2	28	5.4
rp35	Vitellogenin1	40*	Q1LWN2	38	6.3
oe38	Vitellogenin 2	85	Q3T7B3	55	6.6
oe39	Vitellogenin 2	34*	Q3T7B3	49	6.2
oe86	Vitellogenin 2	130	Q3T7B3	18	7.4
c16	Vitellogenin 4	157	Q3T7B1	25	8.3
oe41	Vitellogenin7	371	Q3T7A8	26	8.2
oe69	Vitellogenin7	439	Q3T7A8	26	8.7
c11	Vitellogenin7	144	Q3T7A8	26	6.9

a) For Vtg deriving proteins, calculated MW and pI values are not presented because all of them are Vtg fragments and thus have a smaller MW and different pI compared to the full-length protein.

The Vtg1 deriving fragments identified are represented in Figure 3-5. All Vtg1 Lv1derivatives have molecular weights below 62 kDa and are cleavage products from the full-length Lv1 with a predicted molecular weight of 116 kDa. Most of the Lv2 deriving proteins identified have molecular weights between 25-32 kDa and are consistent with the predicted 26.7 kDa of Vtg1 Lv2. One Vtg1 Lv2 matching protein (c8) has a MW of 38 kDa and seems to include the phosvitin domain. Moreover seven proteins (oe79, c25, c29, c37, r28, r58 and r57) matched with both the Lv1 and Lv2 domain but show a MW of only 28-31 kDa. These proteins may be superimposed in one spot.

Vtg2 is characterised by a larger Lv2 domain (Wang *et al.* 2005) of about 56 kDa. All Vtg2 proteins detected matched the Lv2 domain and most of them (oe38, oe39) had MW of 48-55 kDa, which is consistent with the predicted MW.

Whereas most of the identified full-length Vtg1 Lv2 deriving peptides (c8, c9, c10, c21, c20) were constantly expressed during embryonic development and detectable in gels from 2.5 to 72 hpf embryos, many of the proteins matching to the full-length N-terminal Vtg1 Lv1 were exclusively detectable in younger developmental stages (oe7, oe51, oe27, oe5).

In summary, the identification results confirmed the high abundance of vitellogenin cleavage products in protein extracts from whole zebrafish embryos and illustrate the suitability of using 2-DE analysis to monitor vitellogenin proteolysis processes during embryonic development.

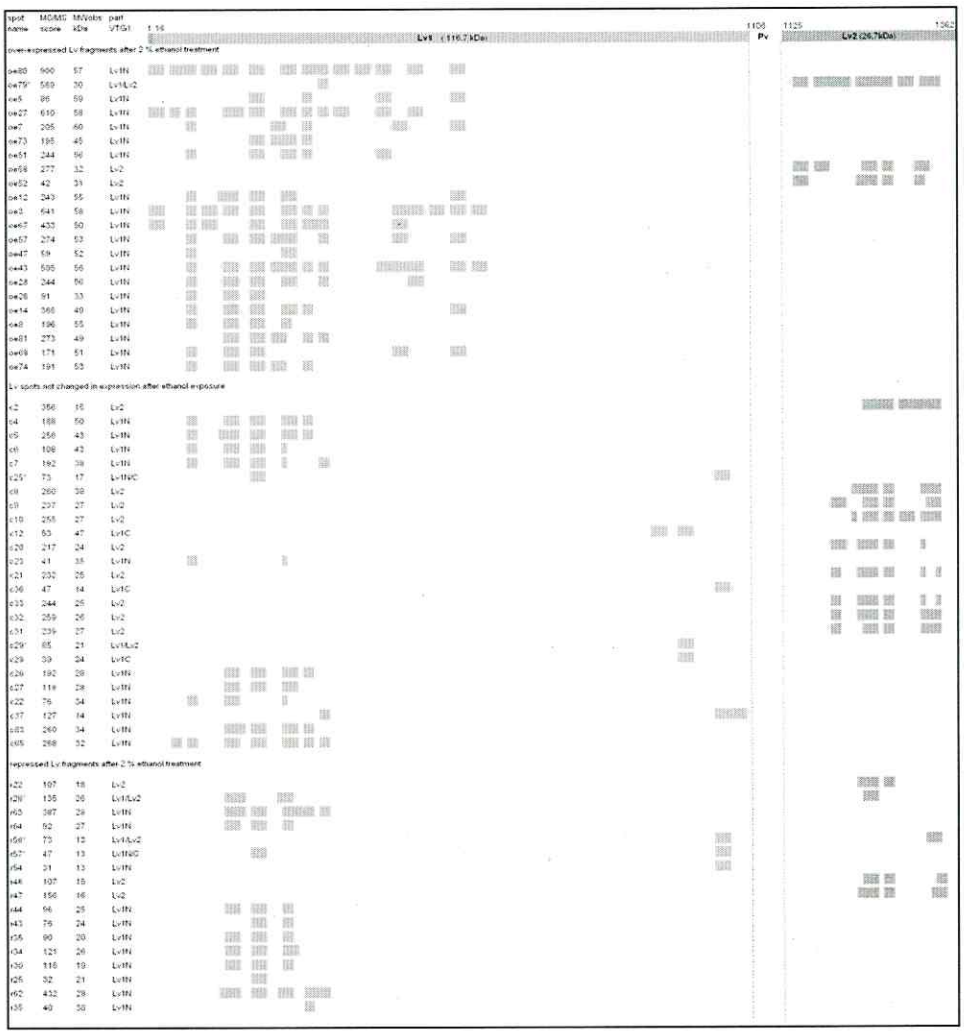


Figure 3-5 Alignment of all identified Vtg1 deriving fragments (shown in Figure 3-4) to the whole Vtg1 protein. Vtg1 consists of 1362 amino acids and includes three domains: Lv1, Pv, Lv2. Labelling of the Vtg fragments is according to an Ethanol exposure experiment (described in Chapter 4) and to Figure 3-4. Moreover, their MASCOT MS/MS scores and observed MWs are shown. For some identified Lipovitellins (Lvs), peptides were identified which would hint to higher MWs of the proteins than observed on the gels. These are marked by asterisks and are supposed to be mixtures of different Lv species in one excised protein spot.

3.2.3 Immunoblot Analysis with Anti-Vtg Antibody

In order to support the MS identification results and to obtain an overview about the amount of vitellogenin derivatives in zebrafish embryo protein samples, immunoblot analysis with a zebrafish anti-vitellogenin antibody was carried out. A polyclonal anti-vitellogenin antibody was chosen ensuring the binding to multiple vitellogenin cleavage products as found on the gels. On the 2-D Western blot shown in Figure 3-6 A which was obtained from protein samples of 2 dpf old embryos, 62 protein spots were detected, which resemble the distribution of the most abundant vitellogenin fragments.

Not all spots which have been identified as vitellogenin fragments could be detected in the Western blot experiment. On reason could be the weak affinity of the antibodies. In addition, the epitopes of the antibodies present in the polyclonal mixture of antibodies are not known. However, the immunoblot analysis supports the finding that many proteins in zebrafish embryo protein samples derive from the yolk precursor vitellogenins.

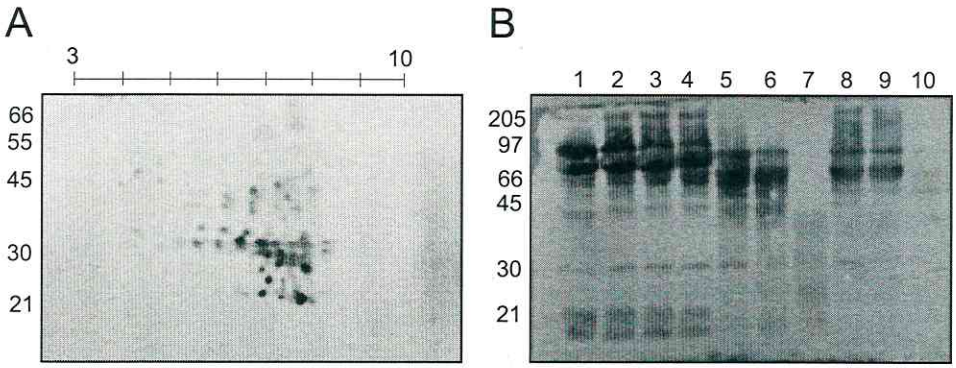


Figure 3-6 Results of Western blot experiments with a polyclonal anti-zebrafish-anti-vitellogenin antibody. (A) Western blot analysis of protein samples deriving from 2 dpf old embryos using 2-DE. Around 62 vitellogenin deriving proteins on 2-DE gels from 2 dpf embryos could be detected. (B) 1D Western blot results from embryonic protein samples at ten different embryonic stages (1: 1.5 hpf, 2: 4.5 hpf, 3: 7.5 hpf, 4: 11 hpf, 5: 25.5 hpf, 6: 31.5 hpf, 7: 2 dpf, 8: 3 dpf, 9: 4 dpf, 10: 5 dpf). Apparent protein bands resemble vitellogenin deriving proteins.

Development-dependent changes in the vitellogenin fragment pattern and the vitellogenin fragment fraction were studied by analysis of protein patterns of different embryonic development stages by 1-D Western blot analysis. The immunoblot assay (Figure 3-6 B) showing vitellogenin cleavage products (lipovitellins) can be directly compared to the coomassie stained proteins of the 1-D SDS-PAGE depicting the whole

protein composition of zebrafish embryos (Figure 3-3). Many of the protein bands in Figure 3-6 B mirror protein bands in the 1-D SDS-PAGE (Figure 3-3) again indicating that most of the embryonic proteins derive from vitellogenin. Consistent to earlier results showing that the protein composition of zebrafish embryos is constant over the first 10 hours (Type I gels, Figures 3-2 A-B) the lipovitellin patterns are also similar over the first 11 hours (Figure 3-6 B, line 1-4). Four lipovitellin complexes at about 100, 80, 30 and below 20 kDa are characteristic for these early embryonic stages. From 25 hpf to 2 dpf a decrease of proteins with higher MW and at the same time an increase of lower MW proteins in the embryonic protein samples (Figure 3-3) as well as the lipovitellin pattern (Figure 3-6 B, line 5-7) can be detected. This indicates cleavage processes of higher molecular proteins to smaller peptides during embryonic development. Surprisingly, an increase in higher MW proteins (Figure 3-3) and also in higher MW lipovitellins (Figure 3-6 B, line 8-9) in hatched 3 to 4 day old zebrafish embryos can be observed. Only a few lipovitellins are found at MW below 45 kDa. The observed protein pattern in 2 dpf old embryos is astonishing (Figure 3-6 B, line 7) since higher molecular bands (at 97 and 66 kDa) appearing in younger (line 6) and older (line 8) stages could not be observed. This could either be due to a failure in the sample (proteolytic degradation) or to biological reasons. However, the protein pattern was reproducible (see Figure 3-3).

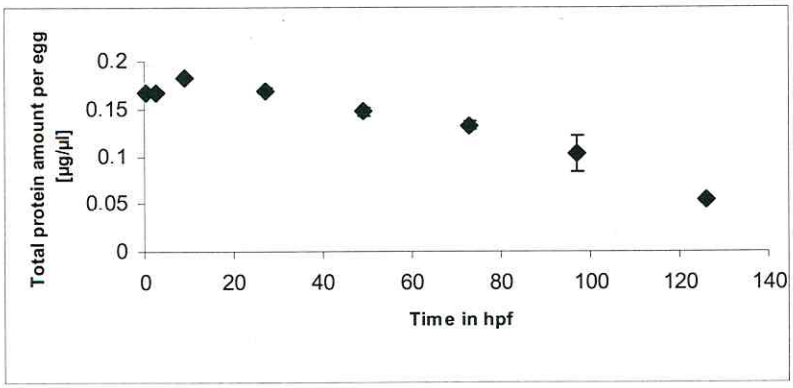


Figure 3-7 Total protein amount (in µg/µl) in the extracted protein samples related to single eggs. Protein concentration in the samples were determined in eight different developmental stages using the detergent compatible BIORAD protein assay based on the method of Lowry *et al.* (1995).

In 5 day old larvae, vitellogenin derivatives were hardly detected (Figure 3-6 B, line 10). At this developmental stage the yolk is almost consumed (Figure 3-2 H) and external feeding starts. Consistent with this, a 2-DE Western blot of 5 dpf old embryos also showed only three lipovitellin spots (results not shown).

To analyse if the decrease in yolk proteins during development can be followed in the total protein amount per egg, the protein concentration in the extracted protein samples at different developmental stages was measured (Figure 3-7). Indeed, after a constant protein concentration in the first 9 hours of development (1.5-2 µg/µl), a continuous reduction of the total protein amount was obtained along the duration of development. Protein samples extracted from 5 dpf larvae showed the lowest measured protein concentration with 0.05 µg/µl. This is in accordance to the finding that only few vitellogenin derivatives were detectable in 5 dpf old larvae and thus, seem to derive from the sum of concentrations of all cellular proteins. In addition, the small standard deviations for each data point in Figure 3-7 show that protein concentrations within developmental stages are quite constant and confirm constant (reproductive) vitellogenin fragment patterns at each developmental stage as observed with 2DE-analyses (Figure 3-2).

3.2.4 Identification of Proteins in Five Day old Larvae

Results from proteome analysis and immunoblot analysis with a polyclonal anti-vitellogenin antibody have shown that the protein samples of larvae differ very much from younger developmental stages due to strong decrease of yolk proteins. To analyse if these results can be confirmed by protein identification, several proteins from 2-DE gels derived from protein samples of 3, 4 or 5 dpf old larvae were excised and identified via ESI-MS/MS.

All identified proteins in the proteome of older embryos or larvae are shown in Table 3-2 and all identified proteins found in the proteome of 5 dpf old larvae are presented in Figure 3-8. Altogether, 36 proteins were identified. Only two of them matched to sequences of vitellogenins. Most of the proteins were muscular proteins (8 actins, 7 myosins, 2 tropomyosins and 1 troponin) with important functions in muscle contraction. Moreover, some proteins important in filament structure (5 keratins, 2 tubulins) could be identified. Next to more structural proteins also one heatshock protein (Hsp60), an ATP-synthase and one transport protein Apolipoprotein could be characterised on the gels.

Mascot scores for identification validation and the observed and calculated MW and pI data, respectively of each identified protein (Swiss-Prot/TrEMBL database) are listed in Table 3-2. Most of the proteins could be identified as *Danio rerio* proteins with high statistically (score values > 100) significance. Moreover, the observed MW and pI values mostly resembled the theoretical expected values. Some differences (e.g. protein

c58, c59, c78, c99, c101) could be due to posttranslational modification or fragmentation events changing the pI and MW of a certain protein.

On the one hand the results show that the ratio of yolk proteins to cellular proteins decreases during development. On the other hand they make clear that validated identification of cellular proteins with applied methods is possible.

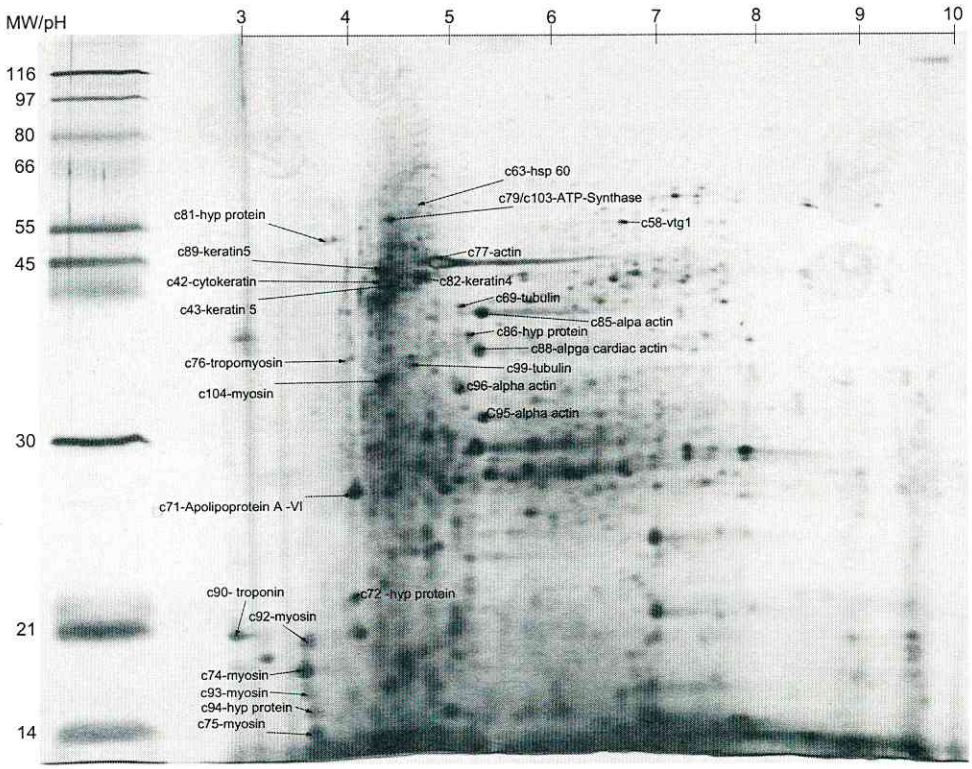


Figure 3-8 2-DE analysis of 5 dpf old larvae and all identified proteins found at this developmental stage are presented. 15 proteins were directly excised and identified from this gel (c79-c104). 11 proteins were excised from gel from younger larval stages and could be matched to the gel of 5 day old larvae (compare to Table 3-2).

Table 3-2 MASCOT Score, protein name, observed or calculated molecular weight (MW) and isoelectric point (pI) for all proteins identified in larvae are listed. The age of the organisms is presented as well.

Spot NAME	*Embryo age*	*MASCOT Score*	*Protein NAME*	*MW obs/cal*	*pI obs/cal*
c79	5 dpf	353	ATP-Synthase	55000 / 54966	5.2 / 5.25
c81	5 dpf	178	hyp protein	47000/99895	9.35
c82	5 dpf	711	keratin4	44000 / 53980	5.4 / 5.34
c85	5 dpf	374	alpha actin	40000 / 42006	5.5 / 5.18
c86	5 dpf	170	hyp prot	38000 / 49685	5.3 / 4.81
c88	5 dpf	236	alpha cardiac actin	37000 / 41969	5.5 / 5.29
c89	5 dpf	513	keratin5	44000 / 58551	5.1 / 5.34
c90	5 dpf	380	troponin c	17000 / 18199	4.0 / 3.96
c92	5 dpf	93	myosin	16500 / 16498	4.6 / 4.39
c94	5 dpf	153	hyp protein	13800/19100	4.4
c93	5 dpf	174	myosin	14000 / 16757	4.4 / 4.39
c95	5 dpf	280	alpha 1 actin	31000 / 41946	5.5 / 5.23
c96	5 dpf	99	alpha actin	32000 / 41956	5.4 / 5.23
c99	5 dpf	139	tubulin	33000 / 52545	5.0 / 4.9
c104	5 dpf	147	myosin	32000 / 48628	4.9 / 5.52
c58	4 dpf	68	vtg1	47000 / 149140	6.6 / 8.74
c59	4 dpf	172	vtg2	48000 / 68994	6.4 / 7.84
c63	4 dpf	170	hsp60	5000 / 61158	5.4 / 5.56
c69	4 dpf	106	tubulin alpha	41000 / 50002	5.5 / 4.93
c70	4 dpf	291	actin	40000 / 41946	5.6 / 5.23
c71	4 dpf	462	Apolipoprotein A-IV	24000 / 29988	4.6 / 4.82
c72	4 dpf	359	hyp protein	19000 / 19110	4.6 / 4.64
c74	4 dpf	394	myosin	15000 / 16498	4.4 / 4.39
c75	4 dpf	293	myosin	12000 / 18853	4.5 / 4.68
c76	4 dpf	253	tropomyosin	36000 / 28608	4.6 / 4.74
c77	4 dpf	673	actin	45000 / 41726	5.3 / 5.3
c78	4 dpf	82	hyp protein	45000 / 99895	4.5 / 9.35
c102	4 dpf	631	krt4	43000 / 53980	5.1 / 5.34
c103	4 dpf	613	ATP-Synthase	55000 / 54966	5.2 / 5.35
c38	3 dpf	66	alpha cardiac actin	43000/ 41969	5.9 / 5.29
c42	3 dpf	210	cytoceratin	44 000 / 46656	4.7 / 5.13
c43	3 dpf	121	Keratin 5	44000 / 58551	4.9 / 5.34
c44	3 dpf	132	alpha actin 1	44000 / 41946	5.1 / 5.23
c55	3 dpf	128	tropomyosin	40000 / 32703	4.5 / 4.7
c56	3 dpf	120	myosin	30000 / 48628	5.1 / 5.52
c101	3 dpf	346	myosin	30000 / 22022	4.7 / 5.54

3.3 Discussion

3.3.1 Protein Pattern Changes During Embryonic Development

The goal of this study was the establishment of a proteomic approach in whole non-deyolked zebrafish embryos (embryonal and larval stages) to enable the detection of environmental stress at the biochemical level. For a successful toxicoproteomics based study, control situations have to be well known. Hence, baseline proteomic data at

different embryonal stages were studied. As protein samples of whole embryos contain yolk proteins (Vtgs) as the major protein group, baseline proteomic data at different developmental stages mainly content information on yolk protein pattern and yolk protein mobilisation processes.

While many publications considered the vitellogenin pathway during oogenesis (Byrne *et al.* 1989, Korfsmeier 1966, Heesen and Engels 1973, Ndiaye *et al.* 2006, Patino and Sullivan 2002, Tyler and Sumpter 1996, Wallace and Jared 1969) there are only a few studies in which yolk protein mobilisation and utilisation after egg fertilisation were studied (Fagotto 1995; Kanaya *et al.* 2000, Sawaguchi *et al.* 2006). Only one study could be found using a proteomic approach for characterisation of yolk protein mobilisation processes (Kanaya *et al.* 2000). For many oviparous animals it is well known that, upon ovarian uptake, the yolk precursor proteins vitellogenins are proteolytically cleaved into three yolk proteins, one phosvitin- and two lipovitellin proteins (Byrne *et al.* 1989, Wallace 1985, Montorzi 1995, Patino and Sullivan 2002). Apart from being amino acid sources, vitellogenin-deriving proteins bind lipids, sugars, phosphates and metal ions and are therefore multidimensional nutrients sources for developing embryos. In order to maintain a regulated supply of nutrients and energy during embryonic development, proteolytic degradation of yolk proteins has to be effectively programmed and a particularly regulated proteolysis can be expected. To date, proteases involved in Vtg-fragmentation are not known but for teleosts, potent enzymes are cathepsins D, B and L (Wallace 1985, Carnevali *et al.* 1999), each supposed to be active at different periods in oogenesis as well as embryogenesis.

The developmental stage dependent processing of Vtgs was reported earlier (Byrne *et al.* 1989, Heesen and Engels 1973, Kanaya *et al.* 2000, Korfsmeier 1966, Tay *et al.* 2006, Tyler and Sumpter 1996, Wallace and Jared 1969) and we could confirm the obvious change of vitellogenin processing during the course of development. Two protein clusters of about 100 and 30 kDa are characteristic for a protein pattern found in embryos younger than 9 hpf. It is likely that the two clusters can be assigned to the main yolk proteins Vtg1 Lv 1 (116 kDa, Cluster 1) and Vtg1 Lv2 (27 kDa, Cluster 2), respectively. Identified proteins from 48 hpf old embryos occurring at the same gel region as proteins from Cluster 2 were matched to Vtg1 Lv2 and could confirm this assumption. The phosvitin-domain was not detectable on the gels because of its low MW (6 kDa). However, the calculated MWs for Vtg proteins from the primary amino acid

structure may differ from the observed MWs on the gels, due to the extended posttranscriptional modifications of Vtgs during vitellogenesis.

Whereas protein pattern from embryos in the early blastula and gastrula stages are generally conserved, dramatic changes in the protein pattern occur within the segmentation period where many morphological alterations in the developing embryos are observed. Most of the cells are differentiated, embryos undergo a shift from axis formation to organs and the somites are developed (Kimmel *et al.* 1995). Therefore the energy and nutrient metabolism may also change resulting in increased yolk protein proteolysis and consequently in alterations in the protein composition. Proteolytic cleavage of yolk proteins is supposed to take place because the number of proteins detectable on gels from 26 hpf old embryos has increased to about 700 (from about 500 in 9 hpf), with all newly appearing spots showing low MW. At the same time the high molecular weight Cluster 1 and Cluster 2 at around 30 kDa, described above, are strongly reduced. While proteins of Cluster 1, referred to as Lv 1, are only detectable in embryos younger than 26 hpf, proteins of Cluster 2, identified as Lv2 proteins, can still be observed in 72 hpf old embryos. These results could indicate that Lv1 proteins are faster utilised than Lv2 proteins. Supporting this, many identified proteins could be matched to the full length Lv2 domain, while no full length Lv1 proteins were observed. Most of the identified Lv1 deriving proteins could be aligned to the N-terminal part of Lv1 indicating that Lv1 is cleaved in an N (Lv1n)- and C (Lv1c)- terminal part. Lv1n and Lv1c proteins were described by Anderson *et al.* (1998) and also proposed by Kanaya *et al.* (2000). However, high amounts of identified full-length Lv1n proteins with around 45 to 66 kDa were only detectable earlier in development (26 hpf). Most of the Lv1n fragments detected in 2 dpf embryos have MWs below 41 kDa (Figure 3) and are therefore assumed to be further proteolytic process cleavage products of Lv1n.

Major alterations of the protein composition were observed in hatched embryos indicating a rearrangement in energy supplying nutrients and yolk protein utilisation. Many new proteins appeared at higher MW and are therefore supposed to be proteins expressed by the embryo itself. Apart from protein spots continuously changing during development, proteins were also observed that were constantly detectable from 2.5 hpf to 5 dpf old embryos, most of them matching to the Lv2 domain. Therefore late utilisation of these proteins in embryogenesis can be assumed. As described by Tay *et al.* (2006) non-conventional Pv-Lv2 fragments could also be detected on the gel indicating manifold and complex proteolytic processes in yolk protein utilisation. Kanaya *et al.*

(2000) modelled a potential proteolytic processing of Vtgs during salmonid development. In zebrafish most steps of yolk protein proteolysis during embryogenesis are unknown and much research effort is still necessary to elucidate yolk protein utilisation during embryonic development.

Although proteins matching to Type1 and Type2 Vtgs were detected, no Type3 Vtg fragments were observed. According to results from Wang *et al.* (2005) most of the identified proteins derived from Vtg1 which shows the highest level of Vtg expression in zebrafish adults. However, proteins matching to Vtg4 and 7 were detected as well. For red sea bream, a dual Vtg system was proposed showing complete hydrolysation of one type of Vtgs during oogenesis for generating a pool of free amino acids, and with the other type of Vtg serving as nutrient carrier for embryonic development (Sawaguchi *et al.* 2006). To date, no such differential utilisation of different Vtgs during oogenesis and embryogenesis was found for zebrafish, but Vtg types (Vtg1-7) missing on the gel could hint to such an explanation.

3.3.2 Ratio of Yolk- to Cellular Proteins Decreases During Embryonal Development

With the established proteomic method in zebrafish embryos, toxic effects/responses at the level of yolk proteins can be studied. To get deeper insights in the *Mode of Action* of toxicants, additional information on the cellular protein level is necessary.

After hatching, major alteration in the protein pattern of the embryos were obtained. Many new proteins appeared at higher MW and are therefore supposed to be cellular proteins expressed by the embryo itself. This process was also described by Tay and coworkers (2006) and was most prominent in protein samples of 5 dpf old larvae. The proteome of 5 dpf old larvae seem to be completely shifted towards different proteins compared to younger developmental stages. Westernblot results could clearly relate this shift to a decrease in the number of vtg derivatives and hence, yolk proteins. This relation is confirmed by protein concentration measurements which show a continuous decrease of the protein amount per embryo/larvae during development. Moreover, MS based identifications of proteins in 2-DE gels derived from 5 dpf old larvae showed, next to many cellular proteins, only 2 Vtg derivatives. Five day old larvae do not possess a visible yolk anymore and start external feeding (Kimmel *et al.*1995) for nutrient supply. Hence, our results confirm and indicate yolk exhausting and complete

yolk protein utilisation in 5 dpf old larvae at the proteome level. Therefore, a toxicoproteomics approach in larvae would allow analysis of cellular proteins without employing manual deyolking techniques which were applied with zebrafish embryos to reduce the content of high abundant yolk proteins in the protein samples (Tay *et al.* 2006, Link *et al.* 2006).

The identification of several proteins in protein samples from 5 dpf old larvae has confirmed the possibility of characterisation of cellular proteins. Most of the identified proteins belong to muscular or cytoskeleton proteins which are considered as ubiquitous proteins and are among the major protein classes identified in environmental studies (Monsinjon and Knigge 2007). As these proteins mainly possess acidic pI values they are responsible for the shift of the protein pattern observed during development. They could hamper the analysis of less abundant proteins which could be important toxicity targets. However, heatshock proteins (Hsps), also considered as higher abundant ubiquitous proteins, are directly linked to toxic stress answer in fish (Iwama *et al.* 1998). Therefore, analysis of patterns of higher abundant house keeping proteins can also give insights in general toxicity pathways (Monsinjon and Knigge 2007). Moreover, if stress induces expression of certain proteins these are increased in their abundance and might become visible in protein samples of exposed organisms. In addition, several methods like immobilised dyes or immunoaffinity methods are described for reduction of high abundant proteins (Ahmed and Rice 2005, Righetti *et al.* 2006, Thulasiraman *et al.* 2005). To apply these techniques to protein samples of *Danio rerio* larvae could be an important task in future to enable a further analysis of treatment specific responses at the biochemical level. However, our results point out, that the investigations of different levels of protein abundances lead to different information on the *Modes of Action* of toxicants. Moreover they support that manual deyolking of embryos leads to protein samples contenting no yolk proteins but again groups of higher abundant house keeping proteins hampering the analysis of lower abundant proteins. It can be concluded that larvae are easier accessible and should be preferred for studying cellular proteins than deyolked embryos because equal cellular protein pattern in the two systems can be assumed (housekeeping proteins).

In summary, the results of this chapter point out that the most abundant protein group in protein samples conducts the information that can be obtained from a toxicoproteomics approach. In developing zebrafish abundance of certain protein groups depends on the developmental stage: early in development yolk proteins and in larval

stages cellular housekeeping proteins are the most abundant protein groups. Hence, ecotoxicoproteomics approaches in embryos lead to information on yolk mobilisation processes and in older larval stages (5 dpf) to information on basal cellular processes. Outcomes of ecotoxicoproteomics with *Danio rerio* embryos and larvae, respectively are analysed and discussed in detail in the subsequent Chapters 4 and 5.

CHAPTER 4

Proteomics Study of Toxic Stress in Early Embryonal Stages of the Zebrafish

4.1 Introduction

Results of Chapter 3 have shown that zebrafish embryos are accessible to a proteomics approach. The proteome of embryos until 4 dpf mainly consist of proteins deriving from the yolk precursor protein vitellogenins. Vitellogenins are ubiquitous yolk precursor proteins which are produced by the female adults and, after inoculation in the oocytes, are proteolytically cleaved to release nutrients essential for the developing embryos. For all oviparous animals, yolk utilisation processes are largely unknown. To our knowledge, only one study published a proteomic based analysis of yolk protein processing processes during embryogenesis of fish (Kanaya *et al.* 2000). This study aimed to understand normal and abnormal developmental processes by 2-DE characterisation of embryonal protein samples at different embryonic periods. A schema of the potential proteolytic processing of vitellogenins in rainbow trout is presented in Figure 4-1.

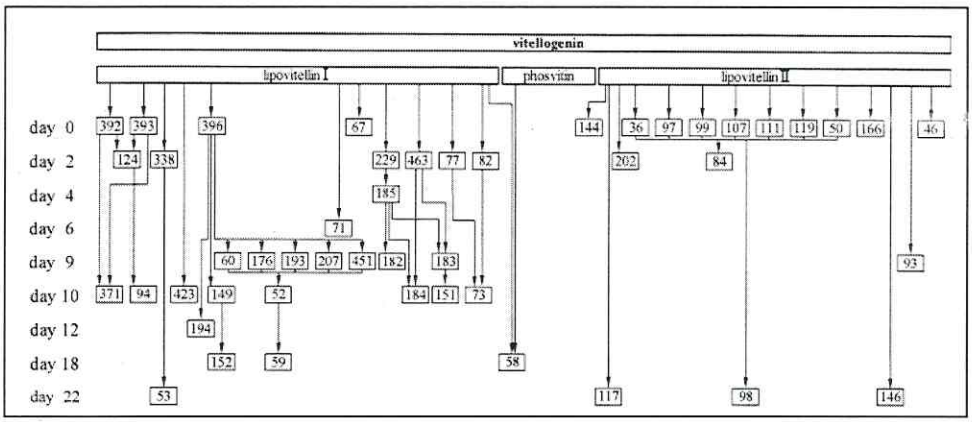


Figure 4-1 Potential proteolytic processing of vitellogenin in rainbow trout published by Kanaya *et al.* (2000). To our knowledge, this is the only study having published a possible processing pathway of yolk proteins in fish. The authors have investigated embryonal protein samples from rainbow trout embryos at different embryonic periods by 2-DE. Numbers of the protein fragments correspond to ID of proteins with sequenced N-terminus.

Although less is known about the exact mechanism of yolk protein proteolysis, many authors have emphasised that a programmed and strict regulated enzymatic processing is crucial for normal embryonal development (Fagotto 1995, Farba and Cerda 2004, Kanaya *et al.* 2000, Kwon *et al.* 2001, Tingaud-Sequeira and Cerda 2007). This was also confirmed by results in Chapter 3 showing developmental stage specific pattern of Vtg fragments. Hence, impairments of yolk protein processing by toxicants could be

expected and might be observable by a toxicoproteomics approach in embryonal stages of the zebrafish.

Schulte and Nagel (1994) have proposed the use of embryonic zebrafish until 2 dpf for acute toxicity testing (DarT, or *Danio rerio* teratogenicity test or fish embryo test) as an alternative to the acute testing with adult fish. Meanwhile, the DarT has replaced the routine wastewater treatment plant effluent testing in Germany (Federal Law Gazette 2005). In contrast to *in vitro* systems, also proposed for the replacement of animal experiments (Routledge and Sumpter 1996), the DarT accounts for toxicokinetic and toxicodynamic processes and hence combines the benefits of *in vivo* and *in vitro* systems - high biological integration and time- and cost efficiency (Eggen *et al.* 2004, Wiegand *et al.* 2000). But until today, the DarT is still not authorised for hazard assessment of chemicals or environmental samples.

Assessment of effects at the molecular level in 2 dpf old zebrafish embryos, allowing the identification of new sensitive toxic endpoints and an understanding of toxicity mechanisms, could support the extension of DarT towards testing of chemicals and allow a more sensitive characterisation of toxic exposure with DarT. This was shown by Muncke and Eggen (2006) who proposed a sensitive system for investigation of endocrine disruption based on gene expression (vitellogenin gene) in zebrafish embryos. This system was derived from a microarray based toxicogenomics study in 2 dpf old zebrafish embryos.

Moreover, the well defined toxic endpoints at the physiological and morphological level in 2 dpf old embryos would allow a direct relation of effects at the molecular level to higher levels of biological organisation and could contribute to the understanding of effect translation from cell to the whole organism.

Therefore, the objectives of the toxicoproteomics study described in this Chapter were to analyse the potential of detecting toxic exposure at the proteome level of 2 dpf old embryos to (*i*) identify whether vitellogenin cleavage patterns change upon toxic exposure and could indicate toxic stress in the embryos at the molecular level and (*ii*) to find new sensitive toxic endpoints (biomarkers) applicable with DarT. Both aims contribute to the overall objective of the work to establish proteomics with developing zebrafish for exploring of mechanisms of toxicity or finding of new candidate biomarkers.

Ethanol is soluble in both aqueous and lipid media, easily crosses membranes and strongly affect zebrafish embryonic development resulting in various abnormalities. These include central nervous system-, axial- and facial malformations, failures in the cardiovascular system and many organs such as eyes, retardation in development, higher mortality and also reduced pigmentation (Baumann *et al.* 1984, Bilotta *et al.* 2004, Blader and Strähle 1998, Carvan *et al.* 2004, Hallare *et al.* 2006, Lockwood *et al.* 2004, Reimers *et al.* 2004). It exerts similar effects in many other embryonic systems. Some genomics and proteomics studies in fish have shown that protein expression is changed upon Ethanol exposure (Damodaran *et al.* 2006, Hallare *et al.* 2006, Lele *et al.* 1997, Liu *et al.* 2004, Mayfield *et al.* 2002). Therefore, Ethanol was chosen as a reference stressor for the present study.

4.2 Results

4.2.1 *Danio rerio* Fish Embryo Test with Ethanol

Preceding the 2-DE analysis of Ethanol effects at the biochemical level, the impact of Ethanol in embryogenesis was studied using the *Danio rerio* fish embryo test (DIN 38415-6 T6, 2001). Ethanol concentrations in a range from 0.03 % and 10 % were tested. At Ethanol concentrations below 0.5 % (v/v) no toxic effects were detected (Figure 4-2 A).

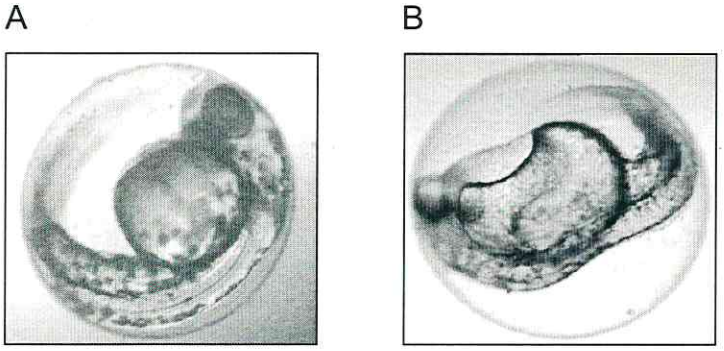


Figure 4-2 Physiological effects of Ethanol to developing zebrafish embryos in comparison to normal development: (A) A normal developed 2 dpf old zebrafish embryo (B) A 2 dpf old zebrafish embryo exposed to 2 % v/v Ethanol for 48 h. The treated embryo shows strong oedema around the heart region and yolk and lacks heartbeat and blood circulation. Moreover a distorted backbone can be seen.

Consistent with results from Hallare *et al.* (2006) and Reimers *et al.* (2004), Ethanol caused impairments in the cardiovascular system like reduced or no heartbeat, no blood circulation and strong oedema around the heart region at higher concentrations

(> 0.5 %). In addition, Ethanol treated embryos showed distorted backbone and pigmentation impairments (Figure 4-2 B). At Ethanol concentrations above 2.5 % most of the embryos were coagulated and only lethal effects could be observed.

Results of the DarT with Ethanol were used to determine the concentrations for the subsequent 2-DE experiment with Ethanol. This should enable the relation of effects at the proteome level towards effects at higher levels of biological organisation. Based on different endpoints two concentration-effect relationships were modelled (Figure 4-3); one including only coagulation of the eggs as lethal endpoint and the other based on different lethal and sublethal endpoints (coagulation, affected heartbeat and/or blood circulation, oedema). The calculated LC50 (50 % lethal concentration) value is 2.52 % (v/v) (0.44 M) Ethanol based on one lethal endpoint and the EC50 (50 % effect concentration) value is 1.22 % (v/v) (0.21 M) Ethanol when more endpoints are included. Similar results were also shown by Reimers *et al.* (2004) who determined an EC50 value of 0.14 M for Ethanol exposure to zebrafish embryos. For the subsequent 2-DE experiment, two Ethanol concentrations were chosen: the low effect concentration 0.5 % v/v Ethanol, at which only 10 % of the embryos showed an effect and 2 % v/v Ethanol, at which all (100 %) of the embryos were affected but only some of them (20 %) coagulated.

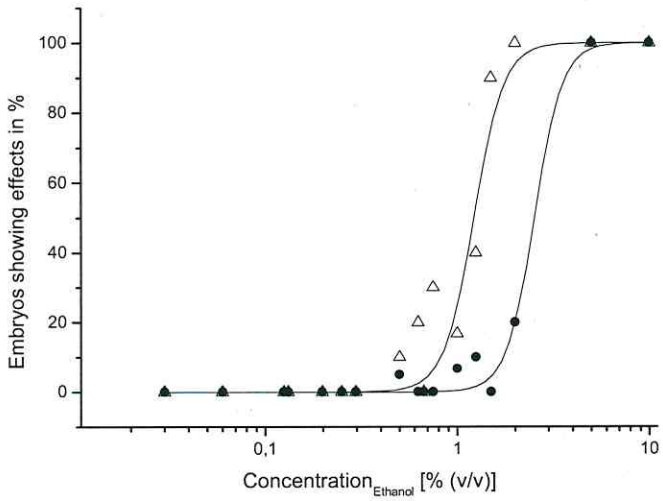


Figure 4-3 Concentration-effect-relationships: Embryos were exposed to different Ethanol concentration for subsequent estimation of two effect-concentration relationships based on different endpoints [coagulation (•) or coagulation + affected heartbeat and/or blood circulation + oedema (Δ)] and a logistic model (parameters estimates: one endpoint: x_0= 2.52±0.148, p= 5.95, R^2=0.991, three endpoints x_0= 1.22±0.058, p=5.54, R^2=0.994).

4.2.2 2-DE Analysis of Zebrafish Embryos Exposed to two Different Ethanol Concentrations

A proteomic experiment was carried out to analyse significantly changed proteins in the zebrafish embryo proteome upon Ethanol exposure. 2-DE gels from protein samples of non-exposed 2 dpf old embryos as well as from embryos treated with two different Ethanol concentrations were prepared. As changes in the protein pattern can be well expected if embryos show microscopically visible toxic effects, 2 % (v/v) Ethanol was selected as the first concentration to be analysed because all (100 %) of the embryos were affected (coagulation or no heart beat and / or no circulation and / or oedema). All coagulated embryos were excluded from the experiment. To assess if a proteomic approach enables sensitive effect recognition at low toxicant concentration, 0.5 % (v/v) Ethanol was chosen as the second tested concentration because more than 90 % of the embryos did not show any toxic effects. In this case, all affected embryos were excluded from the 2-DE experiment.

The 2-DE gels from control and exposed embryo samples were prepared in triplicates. Hence, the 2-DE experiments comprises nine 2-DE gels: three control gels, three gels from 0.5 % Ethanol exposure and three gels from 2 % Ethanol exposure. Using the 100 % spot matching strategy, available from the DECODON software, 740 spots could be distinguished being present on each of the nine received 2-DE gels in the experiment. The 100 % matching strategy ensures the analysis of the same proteins on each gel in the experiment. Spots lying in smear regions of the gels and at the gel-borders were excluded from spot detection.

To get an impression about the variances and hence the reproducibility of the 2-DE experiment, the relative standard distributions (RSDs) of the protein amounts for each of the 740 proteins on the gel were analysed according to the exposure groups. Lower variances (RSDs) would mean a higher reproducibility of the 2-DE experiments and higher RSDs would indicate minor quality of the 2-DE experiments. Frequency distributions of the RSDs for each exposure group are depicted in Figure 4-4. The control group showed the highest mean value with 34.6 % and median-value with 23.5 % compared to all three groups. The mean and median values of the Ethanol exposure groups were below 30 % and below 20 %, respectively. These data indicate that most of the detected proteins have low variances in their amounts within one group. This is confirmed if one considers that 75 % off all data points (for all samples) had a RSD below 50 % (Figure 4-4).

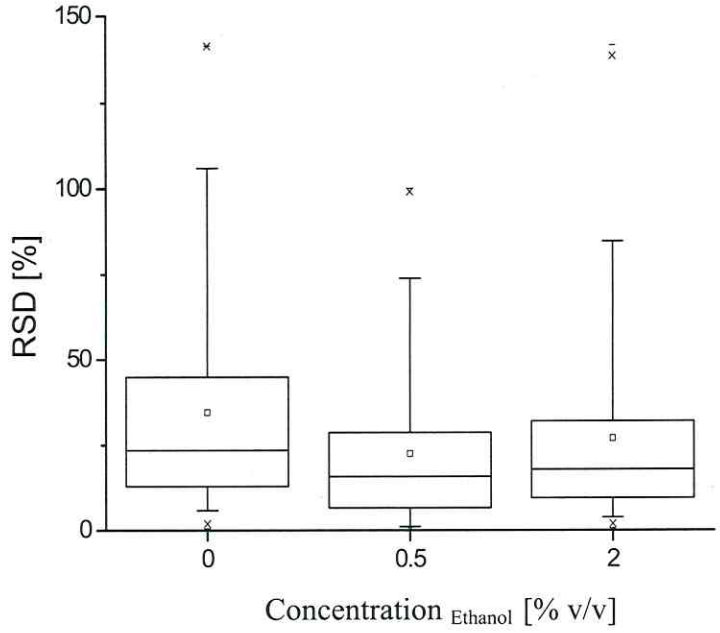


Figure 4-4 Boxblot diagram to show frequency distribution of the relative standard deviations (RSDs) for each of the detected 740 proteins from three 2-DE replicates at different Ethanol exposure conditions: control (0 %), 0.5 % and 2 % Ethanol. On each of the prepared 2-DE gels 740 proteins were detected. For each exposure condition three 2-DE replicates were done (3 x control, 3 x 0.5 %, 3 x 2 %). For each protein on the gels the relative volume was determined and the RSDs of the relative volume of the proteins for the three replicates were calculated. The boxblot shows the frequency distribution of the RSDs of the proteins for the three exposure conditions. The 5 %, 25 %, 75 % and 95 % percentiles are depicted. Moreover the mean-values (mean control: 34.5 %, mean 0.5 % Ethanol: 22.6 %, mean 2 % Ethanol: 27.1 %) and the median values are presented (median control: 23.5 %, median 0.5 % Ethanol: 15.7 %, median 2 % Ethanol: 18.0 %). Minimal and maximal values (x) are also shown.

A typical 2-DE gel obtained from embryos treated with 2 % Ethanol is depicted in Figure 4-6. A control gel from non-exposed embryos is shown in Figure 3-4 (Chapter 3). To facilitate the visual comparison of the two gels, ten proteins were marked as landmarks using asterisks.

Two approaches were applied to compare the 2-DE pattern derived from control or exposed conditions for detection of toxicity related changes in the proteome of the embryos. On the one hand a multivariate statistical method was used to analyse the data and on the other hand univariate spot-to-spot analysis techniques were performed.

Principle component analysis (PCA) was applied to analyse the whole proteome patterns of exposed and non-exposed embryos. The received PCA scores are shown in Figure 4-5. The first component provided sharp separation between two groups: One

group comprises the control gels and the gels deriving from embryos which were exposed to 0.5 % Ethanol but did not have any visible effects. The other group comprises all gels from proteins samples derived from embryos exposed to 2 % Ethanol which all possessed visible toxic effects. With component one (PC1) 47 % of the whole variance between the samples can be explained (Figure 4-5). In contrast to PC3, accounting for the variances of the samples, the second component (PC2) with 14.8 % provided further separation of gels derived from control and 0.5 % Ethanol treated embryos. Control and exposure scenarios thus were distinguishable at the proteome level by PCA.

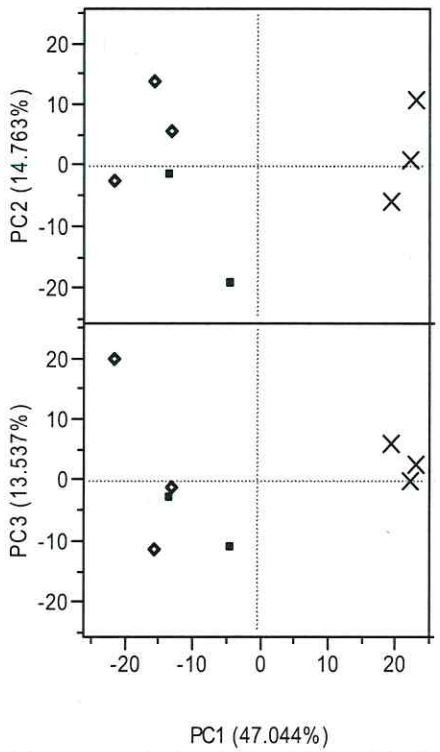


Figure 4-5 PCA scores for protein assessment of control and Ethanol treated embryo samples. Three groups can be distinguished on the scores blot (PC 1 and PC2): control replicates (◊), replicates deriving from 0.5 % Ethanol exposure (■) and replicates from 2 % Ethanol exposure (x). PC3 describes the biological variances and provides no separation of the different treatment groups.

To obtain detailed information about the proteins which have changed related to Ethanol exposure, a univariate spot-to-spot analysis was performed. For this purpose, all spots on the gels derived from Ethanol exposure were examined according to their statistical spread and volume ratio compared with their counterparts on the 2-DE control gels.

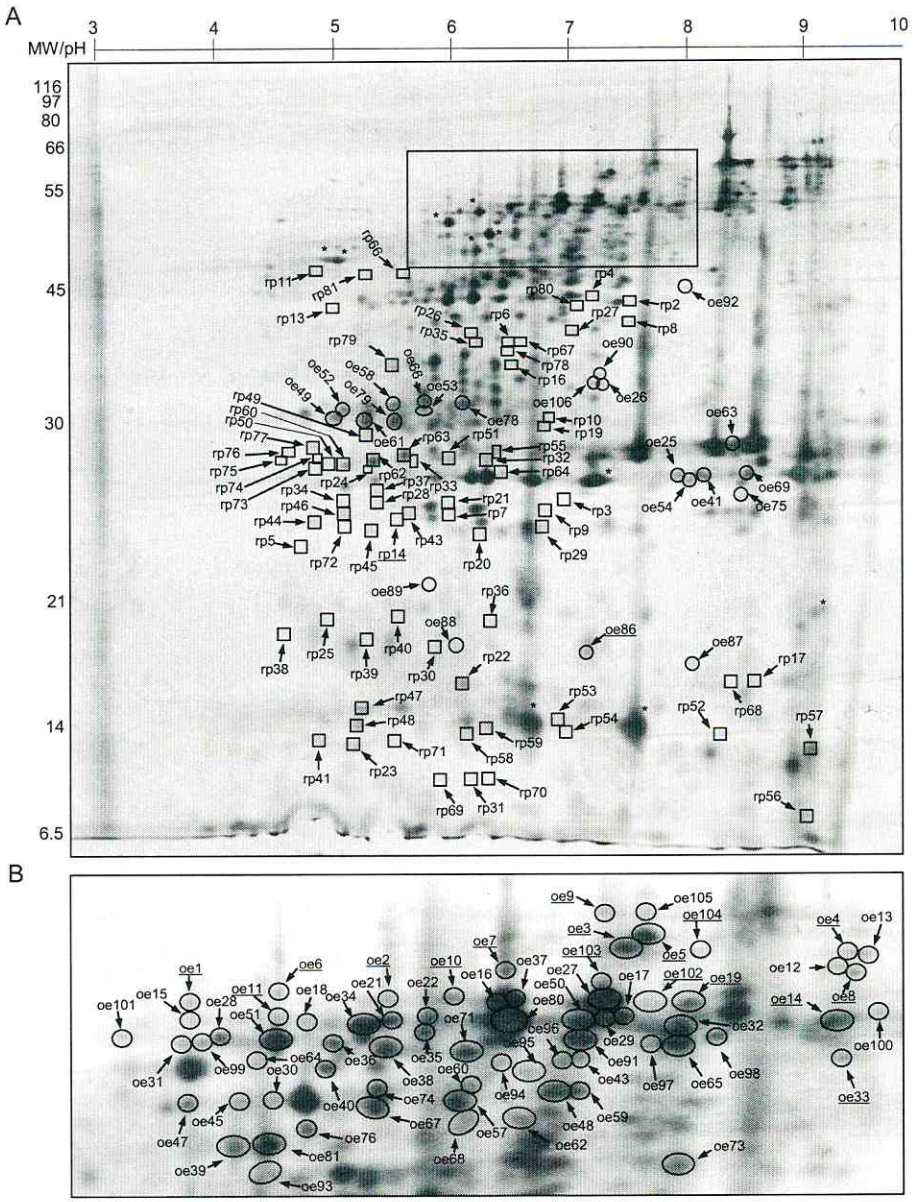


Figure 4-6 2-DE analysis of Ethanol-induced effects in whole zebrafish embryos. (A) A 2-DE gel from 2 dpf embryos exposed to 2 % (v/v) Ethanol is shown. Ninety significantly overexpressed proteins were detected which are indicated as circles on the gel and are named with 'oe' as a prefix. Seventy-four significantly repressed spots are marked by squares on the gel and are nominated with 'rp' as a prefix. (B) Due to narrow distances between spots, the area of the gel containing most of the over-expressed spots is shown in an extended view below the gel. Ten spots marked with asterisks serve as landmarks for easier comparison of the 2-DE gel from exposed embryos depicted in the present figure and the 2-DE gel from non-exposed embryos shown in Figure 3-4 (Chapter 3). All spots that are underlined in the present gel were identified via an ESI MS/MS approach. Identification results are shown in Figure 3-5 and Table 3-1 (Chapter 3).

Only proteins showing at least a significant ($p < 0.05$) two-fold difference in abundance compared to controls, were defined as differently expressed proteins. If the

protein amount after Ethanol treatment decreased more than two-fold, proteins were considered as repressed. Proteins, showing a more than two-fold increase in abundance after Ethanol treatment, were defined as over-expressed. Here the term over-expression refers to elevated amounts of proteins on the gels and implies both new synthesis of proteins as well as fragments derived from already existing proteins by proteolytic processes.

Altogether 90 proteins (12.1 %) were found to be expressed at higher levels after 2 % Ethanol exposure compared with the controls. All detected over-expressed protein spots are indicated by circles in Figure 4-6. Spot IDs including volume-, ratio- and *t*-test-values are summarised in Table A1 (see Appendix). The numbers shown in Table A1 (Appendix) represent the numbers utilised on the gel. Most of the over-expressed proteins appear in two clusters on the gel, one with higher MW values between 45 and 60 kDa (Figure 4-6 B) and one with MW around 30 kDa (Figure 4-6 A). In contrast, most of the repressed spots detected are spread over the gel with all having MW below 45 kDa. In summary, 74 (10 %) significantly and at least two-fold repressed spots (Table A2 Appendix) were detected. These are marked by squares in the gel (Figure 4-6 A).

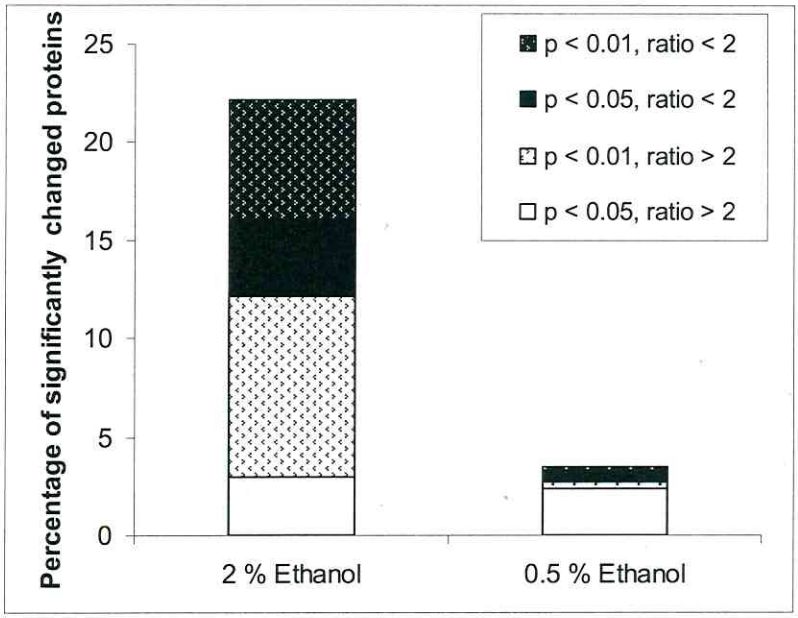


Figure 4-7 Percentage of proteins having significantly changed in their abundance compared to controls after 2 % or 0.5 % (v/v) Ethanol exposure. If abundance of the proteins were more than two-fold increased compared to controls (ratio > 2) they were considered as expressed at higher levels. If abundance of the proteins were more than two-fold decreased (ratio < 2) compared to controls they were considered as repressed. The threshold for significance was 5 % ($p < 0.05$). Among these, all proteins which have highly significantly changed ($p < 0.01$) are indicated.

After 0.5 % Ethanol treatment, less proteins were significantly changed in abundance compared to the 2 % exposure scenario. 20 proteins (2.8 %) were expressed at higher levels in relation to controls. Most of these are also localised at areas of higher molecular weights on the gel. Six proteins (0.8 %) were expressed at lower levels compared to controls.

The percentage of proteins expressed at higher (ratio > 2) or lower (ratio < 2) levels after Ethanol treatment for the two different exposure concentrations are summarised in Figure 4-7. Within the amount of significantly changed proteins, the percentage of proteins being highly significantly changed (α = 1 % or $p < 0.01$) is indicated. In contrast to 0.5 % Ethanol samples showing only 11 % of highly significantly changed proteins, most of the after 2 % Ethanol treatment changed proteins are highly significantly changed (68.9 %).

In order to characterise concentration-dependence of the changed spots, results of the experiments with two exposure concentrations (0.5 % and 2 % Ethanol) were compared. From the 20 significantly and at least two-fold over-expressed spots after 0.5 % Ethanol treatment, 18 proteins also show a significant and at least two-fold difference in expression in the 2 % Ethanol samples. They are marked by grey backgrounds shading in Table A1 (Appendix) and are depicted in Figure 4-8.

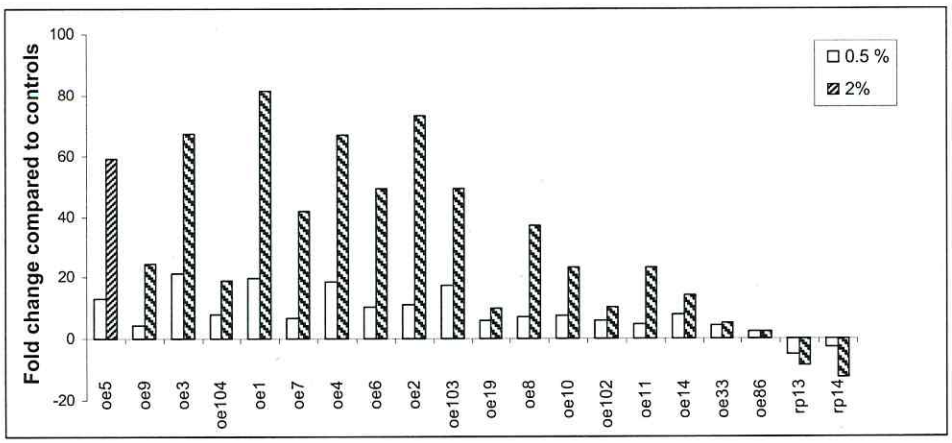


Figure 4-8 Demonstration of 18 over-expressed (oe) and two repressed (rp) proteins which have changed concentration-dependently after two Ethanol treatments (0.5 % and 2 % Ethanol). Abundance is shown in fold-change compared to controls. All depicted proteins, apart from oe86, show increasing effects with increasing toxicant concentrations.

From the 6 repressed spots at a 0.5 % Ethanol treatment, 2 proteins (rp13 and rp14) were also found to be repressed in gels obtained from embryos exposed to 2 % Ethanol. They are marked by grey background shading in Table A2 (Appendix) and are also shown in Figure 4-8.

In summary, 76.9 % of the proteins that were significantly changed after 0.5 % Ethanol exposure were also significantly changed after 2 % Ethanol exposure and therefore are considered as concentration-dependent.

Fifty of the 164 after 2 % Ethanol treatment differentially expressed proteins were excised from the gels and identified by mass spectrometry. While over-expressed spots were taken from gels obtained from 2 % Ethanol samples, repressed proteins were excised from a control gel of non-exposed embryos. The identified spots are shown on the 2-DE gel depicted in Figure 3-4 (Chapter 3). Results of the identification are represented in Table 3-1 (Chapter 3) and Figure 3-5 and have been summarized and discussed previously (Chapter 3). Except one protein identified as Enolase, important in Glycolysis, all of the identified differentially expressed proteins were derivatives of vitellogenins. Whereas many of the identified over-expressed proteins could be aligned to the full length Vtg 1 Lv1n or Vtg1 Lv2, most of the repressed proteins seem to be smaller cleavage products of the full length lipovitellins (Figure 3-5). This give hints to impairments in lipovitellin cleavage processes caused by Ethanol exposure. To assess if the processing of lipovitellins is delayed upon Ethanol exposure the gel obtained from 2 dpf old embryos exposed to 2 % (v/v) Ethanol was visually compared with gels from earlier developmental stages. In fact, many of the spots enhanced in expression upon Ethanol exposure could be recovered from the gel obtained from 26 hpf old non-exposed embryos (compare with Figure 3-2 C).

4.3 Discussion

Teratogenic and embryo toxic effects of alcohol exposure have been described for many vertebrates, including man (Carvan *et al.* 2004). Ethanol generally affects zebrafish embryogenesis resulting in various morphological and physiological effects (Bilotta *et al.* 2004, Blader and Strähle 1998, Carvan *et al.* 2004, Hallare *et al.* 2006, Reimers *et al.* 2004). Genomics and also some protein expression studies analysing Ethanol effects at the biochemical level in zebrafish revealed that gene expression is changed upon Ethanol exposure (Damodaran *et al.* 2006, Hallare *et al.* 2006, Lele *et al.* 1997, Liu *et al.* 2004, Mayfield *et al.* 2002). Therefore, Ethanol was chosen as positive control for analysing

toxicant effects at the biochemical level with a proteomic approach in two day old zebrafish embryos.

Information on variation and hence quality of a method is important for a toxicoproteomics approach to distinguish toxicant specific changed protein patterns from natural variability. The total variation of a method is the sum of biological variability within the samples plus technical measurement errors (Weckwerth *et al.* 2004). The proteome of 2 dpf old embryos mainly contains vitellogenins cleavage products (Chapter 3). Constant proteome pattern therefore strongly depend on regulated enzymatic activity in the yolk. Even if results of Chapter 3 indicated very timed and programmed yolk mobilisation processes, biological variations of yolk protein pattern can still be assumed.

Variation of a 2-DE based proteomic method can include both- qualitative (number of proteins on the gels) and quantitative data (abundance/amount of proteins on the gel) of the protein patterns. Variations seen in different numbers of proteins (qualitative data) on the gels from different replicates were excluded and not considered because we applied a 100 % matching strategy which produces consensus protein patterns valid for all 2-DE gels in the experiment and ensures analysis of the same proteins on each gel in the experiment (Berth *et al.* 2007). Quantitative variations can be studied by analysing the RSDs of the protein amounts of the same proteins in several replicates. The median value for RSDs of all detected proteins in replicates of controls was 23.5%. Hence, most of the detected proteins show constant amounts on the gels even if individual variation was up to 140 %. However, high variable proteins were either low abundant, lay in smear regions on the gel or were localised at the border regions of the gel. The overall variability of only 23.5 % can be taken as a validation of the applied 2-DE method. This finding further supports the assumption of reproducible vitellogenin cleavage patterns in the embryos of the same developmental stage as was observed in Chapter 3 also.

Multivariate pattern and univariate spot-to-spot analysis methods were applied to characterise Ethanol related changes in the protein pattern of the embryos. With both analysis methods differences in the protein pattern of embryos treated with Ethanol compared to controls could be found.

About 22 % of all detected proteins were changed in expression upon 2 % (v/v) Ethanol exposure for 48 h, with 12 % being over-expressed and around 10 % being repressed. The high degree of difference in the proteomes of treated embryos compared to controls hints to a shift in a whole biochemical process rather than the changes of

single proteins. This was confirmed by identification results of many of differentially expressed proteins. All but one identified differentially expressed proteins were found to be derivates of lipovitellins. Thus, it can be concluded that Ethanol exposure causes changes in the proteolysis of yolk proteins during embryonic development. While lipovitellin fragments with high molecular weights appeared at elevated amounts on the gels upon Ethanol exposure, a decrease in low molecular weight lipovitellins was observed. Therefore yolk protein utilisation in embryogenesis seems to be delayed under Ethanol stress. This suggestion was confirmed by finding that many over-expressed spots after 2 % Ethanol exposure in 2 dpf embryos, mainly between 55 and 80 kDa, were also found in protein samples of younger 26 hpf old non-exposed embryos. Interestingly, this observed retardation in development at the proteome level did not clearly manifest at the morphological level. The only effect hinting to retardation was reduced pigmentation.

Impaired and delayed lipovitellin processing during embryogenesis may either be explained by (i) direct inhibition of vitellogenin specific proteases as primary effect or (ii) changed energy metabolism which may result in an altered utilisation of energy resources or (iii) the interference with special lipovitellin functions upon toxicant exposure as secondary effects.

Regarding the first hypothesis, evidence for *in vitro* protease (intracellular lysosomal proteases) inhibition at higher Ethanol concentrations was detected by Mantle *et al.* (1999). Additional inhibition was observed by acetaldehyde which is the product of Ethanol detoxification through alcohol dehydrogenase (ADH). Significant ADH mRNA levels of maternal origin and ADH expression in whole zebrafish embryos were observed indicating that Ethanol metabolism already takes place during embryonic development (Dasmahapatra *et al.* 2001). However, Mantle *et al.* (1999) concluded that direct *in vivo* inhibition of proteases by Ethanol or acetaldehyde is improbable. Moreover in rats, *in vivo* inhibition of cathepsins which are supposed to be involved in the lipovitellin processing (Hiramatsu *et al.* 2002) was not observed (Cook *et al.* 1995).

With respect to the second hypothesis, it has been demonstrated that Ethanol strongly affects the cardiovascular system in zebrafish embryos. Upon 2 % Ethanol exposure, embryos show only a low frequency or absent heart beat and no blood circulation. Consequently, nutrients and oxygen transport within the embryo is strongly impaired. Developing embryos have an increased oxygen demand which, in 2 dpf embryos, could not be satisfied by diffusion processes alone (Finn *et al.* 1995). It may be speculated that Ethanol exposure can lead to hypoxia-like conditions resulting in a shift

from aerobic to anaerobic metabolism processes (Ton *et al.* 2003). Due to impaired oxidative phosphorylation anaerobic glycolysis is the only source for ATP under hypoxic conditions. Ton *et al.* (2003) showed that zebrafish embryos may survive longer under hypoxic conditions by strong up regulation of glycolysis enzymes. The enzyme enolase was found to be unregulated upon 2 % Ethanol exposure, indicating increased glycolysis. Whereas glycogenic amino acids via gluconeogenesis can serve as energy sources for anaerobic glycolysis, fatty acids cannot be used. Therefore lipid metabolism may be altered when embryos are exposed to Ethanol. Lipovitellins are lipid carriers and as a consequence, changed lipid metabolism may be mirrored in impaired yolk protein utilisation. Moreover, down-regulated proteins after Ethanol exposure could indicate the increased need for glycogenic amino acids as energy sources under oxygen deficiency.

Lipovitellins are not only carriers for compounds serving as energy sources like lipids and amino acids but also bind metals, phospholipids and sugars. Therefore impairments of various biochemical and developmental processes aside from energy metabolism can also be expected upon altered lipovitellin proteolysis during embryogenesis. The observed distortion in the embryonal backbone could hint to such an explanation.

Regarding the third hypothesis, assuming special lipovitellin functions, Lai *et al.* (2006) showed that Vtg1 like proteins matching to Lv1 and Lv2, respectively, are components of a DNA binding complex related to DNA repair after UV damage and therefore are involved in DNA protection processes. Direct Ethanol-induced DNA damage was proposed by Kido *et al.* (2006) based on an *in vivo* alkaline single cell gel electrophoresis assay in mice. Consequently, observed Ethanol-caused alterations in lipovitellin utilisation during embryogenesis may also result from an increased need of DNA protection proteins. Otherwise Ethanol exposure could lead to impaired DNA-translation with biochemical or physiological mismatch as consequences.

Changes in the lipovitellin patterns are already caused by 0.5 % Ethanol. Although impairments in the cardiovascular functions of embryos treated with 0.5 % Ethanol were not detectable, chronic dysfunctions of heart and blood circulation, as described in hypothesis two, thus may be indicated at the molecular level. Supporting this, the glycolysis enzymes enolase (oe40) was also found to be slightly increased in expression (ratio 1.6). Hypothesis one, describing the inhibition of proteolytic enzymes, and hypothesis three, discussing the need for DNA protection mechanisms, may also be

consulted for an interpretation of alterations in the lipovitellin proteolytic processes upon 0.5 % Ethanol treatment.

In summary, the proteome analysis of Ethanol-exposed zebrafish embryos allowed the detection of toxic effects at the molecular level. Ethanol exposure could be detected in changed yolk protein patterns due to impairments in the proteolytic cleavage processes of vitellogenin. It can be expected that toxicant exposure, causing impairments at many physiological and biochemical levels, costs energy and lead to an altered energy metabolism which can be directly seen in nutrient usage and hence, in the lipovitellin fragmentation pattern. Altered lipovitellin fragment patterns were detectable in Ethanol-exposed embryos that did not show any microscopically visible effects. Therefore, analysis of vitellogenin cleavage patterns can be used as a sensitive diagnostic tool indicating toxicant stress response in zebrafish embryos early at the molecular level and so the overall fitness of the embryos. For Ethanol, lipovitellin derivatives could be associated with toxic stress. To establish them as indicators for general toxicity analysis in zebrafish embryos further studies are necessary. These includes specificity and sensitivity studies with different classes of toxicants, characterisation of time- and concentration- dependence, phenotypic anchoring and also simplification of the lipovitellin fragment pattern analysis method like immunological detection of specific cleavage products or monitoring of enzymatic activities.

Results of this chapter confirm the possibility of proteomics approaches to give insights in mechanisms of toxicity. Toxic exposure was associated with yolk utilisation processes and with changes in energy/nutrient demand. Thus, the outcome of this work might help to identify a biomarker which enables the monitoring of the overall fitness of an intact organism.

CHAPTER 5

Proteomics Study of Toxic Stress in Zebrafish Larvae

5.1 Introduction

Results of Chapter 3 and Chapter 4 have shown that the proteome of zebrafish embryos is dominated by vitellogenin (Vtg) -derivatives. Proteome analysis with zebrafish embryos thus gives information on yolk utilisation processes. However, the high number of yolk proteins in the protein samples from fish embryos interfere with the detection of more specific information on biochemical pathways and the characterisation of low abundant toxicity targets on the molecular level. This is one of the main arguments why toxicoproteomics analyses with zebrafish by now are nearly confined to adult fish (Bosworth *et al.* 2005, Damodaran *et al.* 2006) and toxicoproteomics approaches with fish embryos have hardly been performed (Shrader *et al.* 2003). Studies with *Danio rerio* embryos or larvae offer some advantages compared to adults. Test organisms can be obtained in high numbers and short breeding time (0-5 days), cultivation is less cost- and time consuming, organism are of small size and allow whole-organism studies and no feeding is necessary which could influence toxicity studies. Therefore, next to the usage of adult fish, strategies are necessary to enable the characterisation of cellular proteins with a proteomic approach in developing zebrafish embryos or larvae also.

One approach pursued the manual deyolking of the embryo and thus the removal of the high abundant Vtg-derivatives (Tay *et al. 2006*, Link *et al.* 2006). This procedure of Vtg removal might generate some kind of stress answer in the embryo which could interfere with the specific stress responses caused by the toxicants analysed with proteomics. Therefore, a more promising approach could be the usage of the developing stage with nearly complete utilisation of the yolk. Results from Chapter 3 have shown that the ratio of cellular proteins to yolk proteins increases during development and that the proteome of 5 dpf larvae barely contains vitellogenins-derivatives. The decrease of yolk in the relation to cell mass during embryonal development was also described by Shrader *et al.* (2003). Kimmel and coworkers (1995) described a produced mouth system and the ability to begin with external feeding for 5 dpf old larvae. This is in accordance with nearly finished yolk consumption at this developmental stage.

Because of that, toxicoproteomics with older larval stages such as 5 dpf larvae seems to be promising for the analysis of proteins with lower abundance than yolk proteins. The well advanced degradation of Vtg-derivatives in 5 dpf larvae could enable the separation of cellular proteins and thus give insights in various protein levels and biochemical pathways without using adult zebrafish or manually removing of the yolk.

Moreover, compared to younger developmental stages, increased activity of detoxification processes can be assumed in larvae (Wiegand *et al.* 2000) and might be investigable. As larvae have all organs developed, are not surrounded by a chorion anymore and are free swimming, toxicodynamic and -kinetic processes in larvae might be more similar to adults compared to embryos and all detected toxicity processes at the molecular level in larvae could be similar to general toxicity mechanisms occurring in adult organisms. This work could thus contribute to the replacement of experiments with adult fish.

Therefore, this chapter aims to establish and to characterise the potential of proteomics with zebrafish larvae. The objectives addressed in this chapter were (*i*) to investigate if toxic stress can be detected in the proteome of treated larvae (*ii*) to test if exposure with different toxicants can be discriminated at the molecular level and (*iii*) to identify possible candidate protein biomarkers indicating exposure against toxicants.

Preceding the toxicoproteomics studies, the sampling point and toxicant exposure conditions according to DarT had to be established for larvae. Rotenone, Dinitro-o-cresol (DNOC) and Diclofenac (Table 2-1) were chosen as reference toxicants with different *Mode of Actions* to study the potential of ecotoxicoproteomics with zebrafish larvae. Whereas they have different molecular toxicity targets, both, Rotenone and DNOC, affect the respiratory chain and were chosen because of their action in an important primary metabolic pathway by decreasing ATP synthesis. The insecticide Rotenone binds, as a primary toxicity target, to the PSST-subunit of complex 1 of the electron chain and inhibits the oxidative phosphorylation (Schuler and Casida 2001). The insecticide and herbicide DNOC acts as decoupler of the mitochondrial oxidative phosphorylation (Pelfrene 2000). The pharmaceutical Diclofenac, as anti-inflammatory drug, inhibits the cyclooxygenases Cox-1 and Cox-2 (Vane and Botting 1998) and was chosen as a toxicant affecting a secondary biochemical pathway.

5.2 Results

5.2.1 Optimisation of 2-DE Method Concerning Age of Larvae

The aim of this chapter was to enable toxicoproteomics with a larval stage of zebrafish which combines both, a low number of yolk proteins in the protein samples and

the avoidance of external feeding. 5 day old larvae have developed mouth and could begin with external feeding (Kimmel *et al.* 1995). Furthermore, results from Chapter 3 have shown that the shift in the 2-DE protein pattern from yolk proteins to cellular proteins is clearer in 5 than 4 day old larvae and that many yolk proteins detected in younger stages disappeared in the proteome of 5 dpf old larvae. 6 days after fertilisation, without feeding, larvae die from starvation. Hence, 5 day old larvae were chosen for the experiments. However, at 120 hpf still some vitellogenins fragments could be identified in the protein samples and it can be concluded that yolk utilisation is ongoing within the fifth day of development.

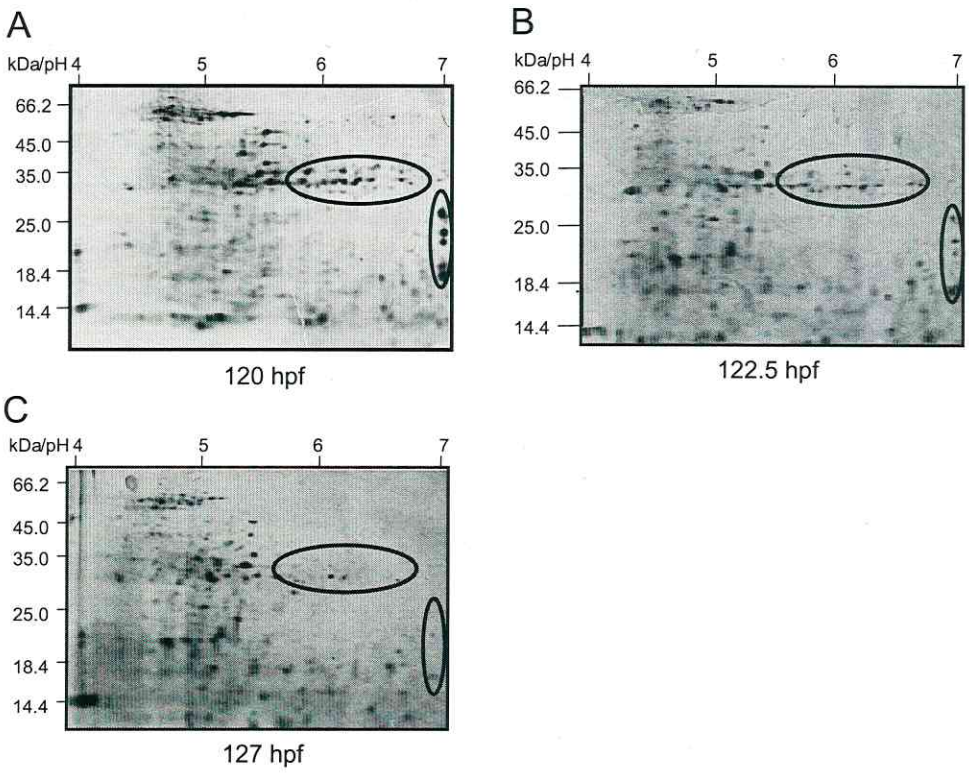


Figure 5-1 Three 2-DE gels from protein samples of zebrafish larvae at different time-points in the fifth day after fertilisation ((A) 120 hpf, (B) 122.5 hpf and (C) 127 hpf). An area which shows high variability in the proteome pattern depending on the measured time points is indicated by circles.

As a first step towards a proteomics approach in *Danio rerio* larvae, the exact age of larvae within the fifth day of development had to be selected for the 2-DE approach which allows both, low number of vitellogenins derivatives and at the same time avoidance of starvation stress.

To characterise the variability of larval protein patterns older than 120 hpf (5 dpf), protein samples deriving from larvae at three different time points within the fifth day after fertilisation were studied. The protein patterns from larval proteomes at 120 hpf, 122.5 hpf and 127 hpf are depicted in Figure 5-1. To avoid starvation, no samples from larvae older than 127 hpf were characterised. Although most proteins in the three protein samples show constant abundance at the different measured time points, two areas on the gels presented in Figure 5-1 A-C were quite variable and are indicated on the depicted gels. A strong decrease of the number and amount of the detected proteins in these certain areas were observed. If proteins in these areas are related to identified proteins occurring in the same 2-DE gel areas from protein samples of 2 dpf old embryos (Figure 3-4, Chapter 3), it can be concluded, that most of them are vitellogenins derivatives. As the number and abundance of these have strongly reduced in samples from 127 hpf old larvae (Figure 5-1 C), larvae were sampled in this age of larvae for all following toxicological and toxicoproteomics analyses.

To characterise the biological variances in the proteome of larvae sampled at 127 hpf and originating from different spawning events, three biological replicates (referred to as biological sample 1-3) were analysed. From each biological replicate, three technical replicates were performed.

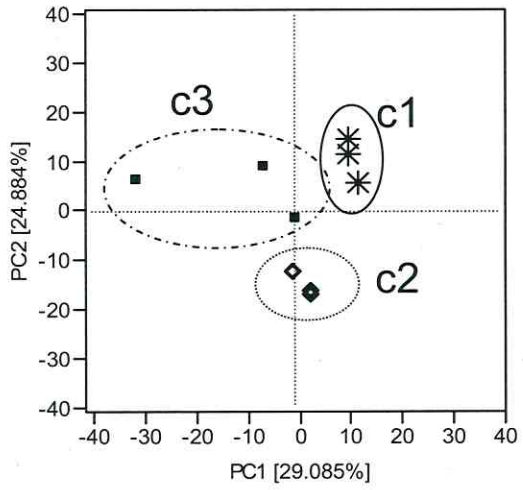


Figure 5-2 PCA scores for protein assessment of 9 different larval protein samples: Three biological 2-DE replicates were analysed (c1, c2, c3) and from each biological replicate three technical 2-DE replicates were performed. The biological replicates derived from larvae from different spawning events on different days.

For each biological replicate 40 fish eggs from different spawning events on different days were incubated collectively under control conditions for 127 hpf and pooled for analysis. All 2-DE gels from biological and technical replicates were loaded as one 'experiment' into the DECODON software and the 100 % matching strategy, ensuring the same protein pattern on each gel, was applied. In total, 600 detected protein spots were included in the analysis.

A multivariate pattern analysis was performed to compare the different biological and technical replicates. The quantification data of the 600 proteins from the 9 samples (three biological and each three technical replicates) were characterised by PCA. The PCA score of PC1 and PC2 distinguished three sample groups (Figure 5-2). Each group comprise the technical replicates of one biological replicate. Hence, technical variances were lower than biological variances and protein samples from larvae originating from different spawning events could clearly be distinguished. It was concluded, that for all toxicoproteomics experiments untreated controls from the same spawning event as the treated samples should be included.

5.2.2 Analysis of Morphological and Physiological Effects in Zebrafish Larvae Treated with DNOC, Rotenone and Diclofenac

Toxicoproteomics allows characterisation of toxic effects at the molecular level. To enable the investigation of toxicity relevant concentrations with the toxicoproteomics approaches, effects at higher biological organisation levels were characterised in the larvae for all tested chemicals. Subsequently, toxicity relevant concentrations were deduced from the established concentration-effect relationships. The knowledge of morphological or physiological effects is also necessary for the characterisation and interpretation of effect translation processes from the cellular to organismic levels.

Effect detection in larvae was done parallel to the fish embryo test (DarT) introduced by Nagel (2002) in terms of test regime, exposure time and toxic endpoints and was referred to as the fish larvae test. The larvae were treated with the toxicants for 48 hours. Incubation in the toxicant solutions started at around 79 hpf when most of the larvae were already hatched after collective cultivating of the fish eggs under control conditions. 48 hours after treatment with the toxicants, larvae were analysed for morphological or physiological effects using inverse microscopy. In addition, fish embryo tests with the three substances were performed, to enable the comparison of

effects in embryos and larvae. All detected effects in embryos and larvae, respectively, treated 48 h with DNOC, Rotenone or Diclofenac are summarised in Table 5-1.

Table 5-1 Summary of all detectable lethal and sublethal effects after 48 h treatment with three substances DNOC, Rotenone and Diclofenac of embryos and larvae. Occurred effects at the different tested concentrations and their frequency are summarised in the appendix (Tables F to K).

Effect	*Diclofenac*		*DNOC*		*Rotenone*	
	embryos [a] (2 dpf)	larvae[b] (5 dpf)	embryos[c] (2 dpf)	larvae[d] (5 dpf)	embryos[e] (2 dpf)	larvae[f] (5 dpf)
Lethal						
coagulation	+	+	+	+	+	+
no heartbeat	+	+	+		+	
no blood circulation	+	+	+	+	+	+
Sublethal						
retardation in development[#]	+		+		+	
affected heart beat[#]	+		+		+	
early hatching[#]			+			
distorted backbone	+	+				
oedema	+	+	+	+	+	
pigmentation impairments[#]	+		+		+	

Tested concentration range:
a 0.0014-0.184µM c 0.25-252µM e 0.0025-1267.74 nM
b 0.00289-0.184 µM d 0.05-50.5µM f 0.253-2535.4 nM
sublethal effects which could be discriminated in embryos but which are not adequate for larvae

All three substances caused lethal (coagulation, lack of heartbeat and blood circulation) and sublethal effects like retarded development, decreased heart frequency or oedema in heart or yolk region in the embryos. Most of them occurred concentration-dependent after treatment with all substances and are shown in Figure 5-3 A and B. Moreover, DNOC exposure resulted in earlier hatching of the embryos and Diclofenac lead to malformations in the backbone.

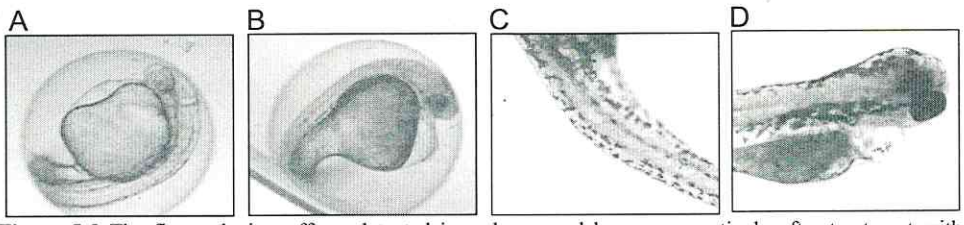


Figure 5-3 The figure depicts effects detected in embryos and larvae, respectively after treatment with toxicants. A 2 dpf old embryo showing retarded development after treatment with Rotenone (A) and a 2 dpf old embryo with impairments of the cardiovascular system and oedema in the heart region caused by DNOC exposure (B) are shown. Two typical sublethal effects in larvae, malformation of the backbone (C) and oedema in the heart region (D) caused by Diclofenac are depicted as well.

All lethal and sublethal effects which occurred concentration-dependent and were quantifiable (not pigmentation impairment or backbone distortion) were consulted for modelling of the concentration effect relationships depicted in Figure 5-4. The EC_{50} values and slopes estimated with the logistic model y = 100 + (-100) / (1 + (x/x0)^p),

with x the concentration in % (v/v), x_0 the median effect concentration (EC_{50}) and p the slope, are summarised in Table 5-2. The potency of Rotenone (EC_{50} = 0.0188 µM) was highest compared to DNOC (EC_{50} = 1.73 µM) and Diclofenac (EC_{50} = 9.16 µM). No large differences in the slopes of the modelled concentration-effect curves for all three substances were observed (Rotenone 5.15, DNOC 4.55 and Diclofenac 3.55).

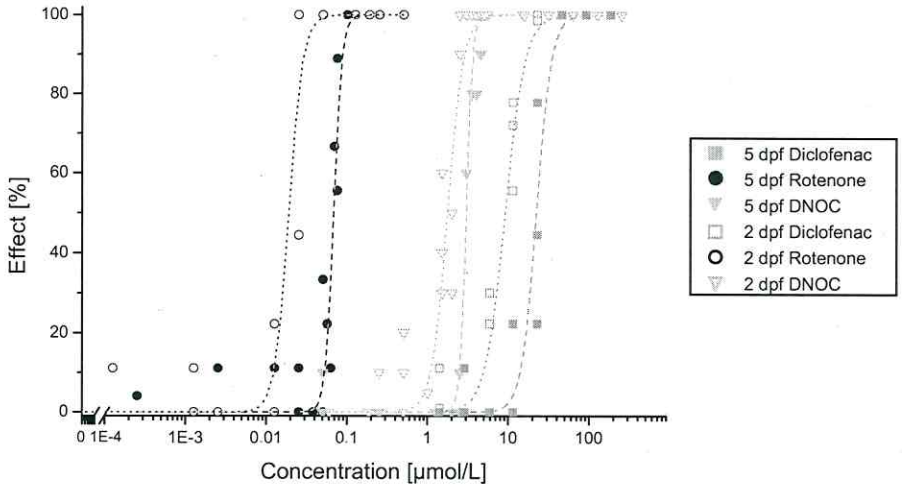


Figure 5-4 Concentration-effect-relationships modeled with a logistic model from observed sublethal and lethal effects in embryos and larvae, respectively, after 48 h exposure against Rotenone, DNOC and Diclofenac are depicted. Parameter estimates are summarised in Table 5-2.

In contrast to embryos, the primarily observed effects in larvae after treatment with Rotenone and DNOC were lethal effects including coagulation and lack of blood circulation and heartbeat (Table 5-1). In addition, Diclofenac caused also sublethal effects like oedema in the heart region and distortions in the backbone as depicted in Figure 5-3 (C + D). Retardations in development or impairments in pigmentation, as detected in embryos, could not be observed as sublethal effects in larvae. Since consistent sublethal effects in larvae were only detectable for Diclofenac, only data points deriving from lethal effects were consulted for establishing of the concentration effect relationships for DNOC, Rotenone and Diclofenac exposure (Figure 5-4) in zebrafish larvae. Estimated EC_{50} values and hill slopes are summarised in Table 5-2. As was also observed for embryos, Rotenone exerted the highest potency in larvae (EC_{50} = 0.068 µM) followed by DNOC (EC_{50} = 3.007 µM) and Diclofenac (EC_{50} = 23.076 µM). The hill slopes obtained in the concentration-effect-relationship models for Rotenone and

DNOC exposure were quite high (8.75 and 12.3, respectively) and exceeded the one estimated for Diclofenac (4.74) exposure in larvae.

Table 5-2 The table summarises the parameter estimates (EC_{50} values and hill slopes) for the logistic two parameter model fitted to data points deriving from observed sublethal and lethal effects in embryos and larvae, respectively, occurring after exposure against three toxicants: Rotenone, DNOC and Diclofenac.

	2 dpf		*5 dpf*	
	EC_{50} [µM]	P	EC_{50} [µM]	P
Rotenone	0.019±0.00	5.15±1.53	0.068±0.00	8.750±2.25
DNOC	1.67±0.06	4.55±0.71	3.007±0.05	12.311±1.80
Diclofenac	9.16±0.42	3.55±0.48	23.076±1.08	4.746±1.65

Estimated EC_{50} values for the three substances clearly indicate that larvae are less sensitive than embryos (Table 5-2 and Figure 5-4). Larvae show a three times lower sensitivity against Rotenone and about two times lower sensitivity against DNOC and Diclofenac compared to embryos. Except for Diclofenac, the estimated Hill slopes were higher for concentration effect curves from larvae than from embryos. The primarily observed effects in larvae were lethal effects like coagulation. Embryos in contrast, showed multiple sublethal and lethal effects. However, all effects occurring in larvae also occurred in the embryos (Table 5-1).

5.2.3 Toxicoproteomics Approaches with Zebrafish Larvae Using three Toxicants Rotenone, DNOC and Diclofenac

Three reference toxicants with different *Mode of Actions*, Rotenone, DNOC and Diclofenac, were applied for characterisation of the potential for toxicoproteomics studies with zebrafish larvae. Only exposed larvae not showing any physiological or morphological effects were included in the 2-DE experiments to ensure the characterisation of the predictive capability of the approach and an easier access to the interpretation of the data obtained from the proteomics studies. Prediction capability hereby refers to the detection of toxic exposure at the molecular level at toxicant concentrations which do not lead to the occurrence of physiological or morphological effects. Hence, preceding the protein isolation step, the exposed larvae were controlled for effects and all larvae showing microscopically visible sublethal or lethal effects were excluded from the proteomics analysis.

Exposure concentrations for the toxicoproteomics experiments were deduced from the concentration effect relationships depicted above (Figure 5-4). For all three substances, the estimated EC_{10} concentrations were chosen for the proteomic studies ($EC_{10Rotenone}$ = 0.05 µM, EC_{10DNOC} = 2.8 µM, $EC_{10Diclofenac}$ = 12.6 µM). To enable the

characterisation of dose-dependence, the modelled EC_{01} concentration for Diclofenac (6.3 µM) and the modelled EC_{40} concentration for DNOC (3.5 µM) were tested as well.

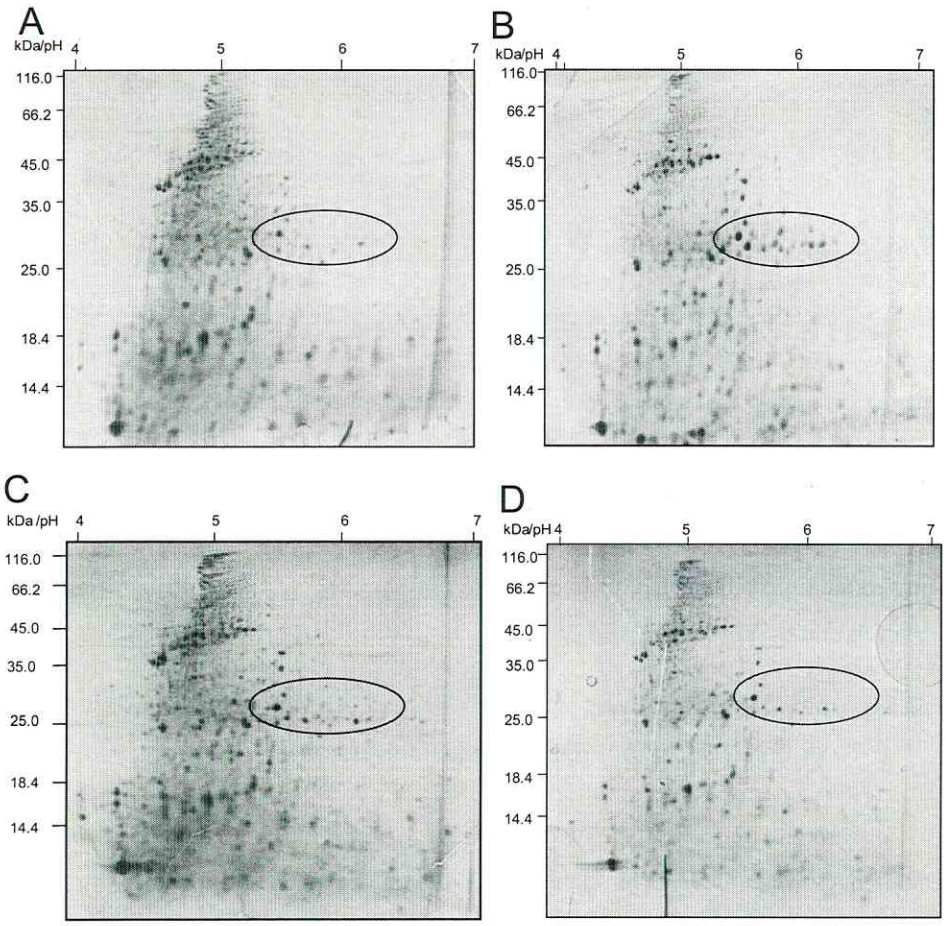


Figure 5-5 2-DE gels derived from larval protein samples. Each depicted gel represents proteome patterns from larvae treated with different toxicants. In (**A**) a 2-DE gel from larvae cultivated under control conditions is depicted. The gel presented in (**B**) shows the proteome from larvae treated with the EC10 concentration of Rotenone, the gel in (**C**) from larvae treated with DNOC (EC10) and the gel in (**D**) from larvae treated with the EC10 concentration of Diclofenac. All protein samples were separated under the same 2-DE conditions: Immobiline strips pH 4-7 in the first and 14 % PAA gels in the second dimension.

Typical larval proteome pattern from control larvae and larvae treated with the EC10 concentrations of Rotenone, DNOC and Diclofenac, are depicted in Figure 5-5. The encircled areas indicate a region on the gels which shows high variances between the four tested conditions. After Rotenone treatment, many new proteins spots compared to controls appeared in that region. Whereas proteome pattern of Diclofenac treated larvae resemble control protein pattern in that region, for DNOC treated larvae also some new appearing proteins in that gel area were observed. Interestingly, the encircled area is

similar to the 2DE-area which was found to be of higher variability in proteome pattern of larvae younger than 127 hpf (see Figure 5-1) and seems to contain vitellogenin derivatives (Chapter 3 Figure 3-4).

Two approaches were applied to compare the 2-DE pattern derived from control or exposure conditions for detection of toxicity related changes in the proteome of the larvae (Monsinjon and Knigge 2007). On the one hand multivariate statistical methods were used to analyse the whole protein patterns and on the other hand univariate spot-to-spot analysis techniques were performed for a detailed characterisation of differentially expressed proteins.

Multivariate Pattern Analysis of the Proteomics Experiments

Principle component analysis (PCA) was applied to analyse the whole proteome pattern of exposed and non-exposed embryos. PCA was performed with the quantification data of all detected proteins in all 2-DE gels in one experiment. The received PCA scores for the tested toxicants are shown in Figure 5-6. The PCA distinguished between control and treatment groups for all tested substances, Rotenone, DNOC and Diclofenac. The separation between the groups was mostly provided by the principle component 1(PC1).

For Rotenone, the first component leads to a very sharp separation between the control and treatment groups and explained 54.6 % of the variances of the samples (Figure 5-6 A). PC2 (16.242 %) and PC3 (14.101 %), in contrast, do not contribute information to distinguish between control- and Rotenone exposure- protein patterns but account for variances within the treatments.

A separation of three treatment groups is shown by the PCA scores deriving from larval protein samples after DNOC exposure (Figure 5-6 B). Whereas PC1 separates between control and treatment samples (32.4 %), PC2 provides the separation of different exposure concentrations (32.43 %). PC3 (11.07 %) accounts for variances within the samples but contributes no information to distinguish between the different treatment situations.

For Diclofenac, the PC1 provides a separation between control and treatment conditions (Figure 5-6 C). The two tested concentrations (EC_{01} and EC_{10}) groups are not separated with PC1 but are distinguishable by PC3 (14.74 %) clearly

separating samples from EC_{10} treatment and EC_{01} treatment. PC2 shows no differentiation between the treatments but contains information on sample variances.

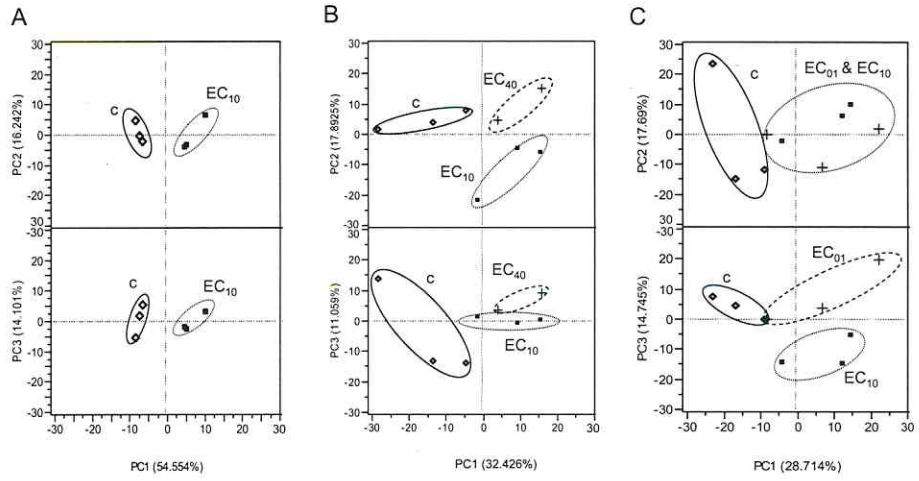


Figure 5-6 PCA scores (PC1, PC2, PC3) for protein pattern assessment of control and treated larvae for three different toxicants (A) Rotenone, (B) DNOC and (C) Diclofenac are depicted. For all toxicants, the EC_{10} concentration was tested. For DNOC and Diclofenac the estimated EC_{40} and EC_{01}, respectively concentrations were analysed additionally. Control groups are indicated with c. For all treatment groups, three independent replicates were performed. All concentrations of one substance were tested in one experiment and are derived from larvae of the same spawning event.

In summary, principle component analysis shows that control larval protein pattern differ from larval proteome pattern after treatment with all tested toxicants and all concentrations investigated. Exposed larvae showing microscopically visible effects were excluded from the 2-DE analysis. Hence, alterations in the proteome pattern can be detected in healthy looking organisms (by microscopical inspection) at toxicant concentrations that do not cause effects at higher biological organisation levels. Compared to classical approaches (e.g. physiological or morphological effect screening), proteome analysis thus enables a more sensitive detection of effects at lower toxicant concentrations.

Univariate Spot-to-spot Analysis of the Proteomics Experiments

Univariate spot-to-spot analysis methods were preformed as a second step in the analysis of the 2-DE experiments to obtain detailed information about single proteins changed in expression or abundance after toxicant treatment. Spot-to-spot analysis is based on the comparison of quantification data of each protein on the gels for all treatment samples of one 2-DE experiment. In accordance to Monsinjon and Knigge

(2007), a protein was defined as differentially expressed if it shows a significantly ($p < 0.05$) at least two fold changed abundance compared to controls. Testing for significance was done applying the Student`s *t*-test available in the Delta DECODON software. If the proteins occurred with higher abundances on the treatment gels than on the control gels, they are considered as over-expressed (oe). If proteins possess decreased amounts on the treatment gels compared to controls, they are referred to as being repressed (rp).

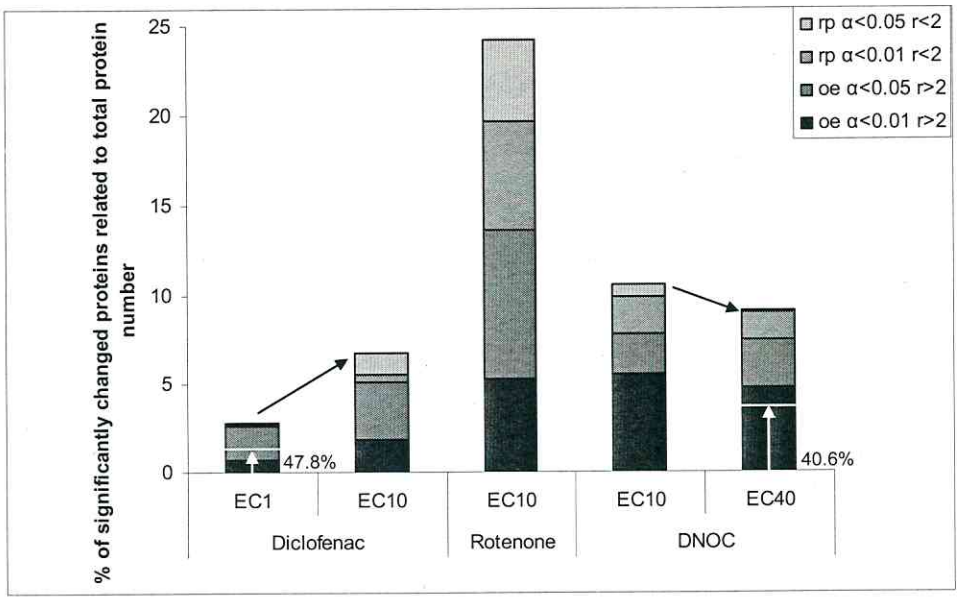


Figure 5-7 Summary of results from spot-to-spot analysis of toxicoproteomics experiments with Diclofenac, Rotenone and DNOC. For all tested concentrations the percentages of proteins, related to all detected proteins in the 2-DE experiment, are depicted which show significantly ($p < 0.05$) and a minimum of two-fold changed expression levels compared to controls. Proteins showing an at least two-fold increase in abundance compared to control ($r < 2$) are considered as overexpressed (blue), all proteins showing an at least two-fold decrease in abundance compared to controls are considered as repressed. The Student`s *t*-test was applied to test for significance. All proteins showing a high-significant expression change ($p < 0.01$) are indicated as well. Black arrows show the relation of the number of differentially expressed proteins to different concentrations of the same toxicant. The white arrows indicate the percentage of proteins which have shown a significant change in expression rate at both tested treatment concentrations of the same compound. 47.8 % of the changed proteins at EC_{01} treatment with Diclofenac were changed in expression also after EC_{10} treatment. 40.6 % of the differentially expressed proteins after EC_{40} treatment with DNOC showed expression changes also after EC_{10} treatment.

Table 5-3 Summary of results from spot-to-spot analysis for all performed toxicoproteomics experiments: Diclofenac (EC_{10} an EC_{01}), Rotenone (EC_{10}) and DNOC (EC_{10} and EC_{40}). Numbers and percentage (in relation to total protein number on the gel) of significantly ($p < 0.05$) and high-significantly ($p < 0.01$) at least two-fold changed proteins are indicated in the table. Data are sorted for overexpressed (oe, ratio sample/control > 2) and repressed (rp, ratio sample/control < 2) proteins. In addition, the sum or percentage of all changed proteins for each condition is shown (rp + oe).

Toxic Compound	*Total number of Proteins detected on the gels*	*p value*	*EC*	*rp [number of proteins]*	*rp [%]*	*oe [number of proteins]*	*oe [%]*	*rp + oe [number of proteins]*	*rp + oe [%]*
Diclofenac	814	0.05	EC_{10}	14	1.7	41	5.0	55	6.8
	814	0.01	EC_{10}	4	0.5	15	1.8	19	2.3
	814	0.05	EC_{01}	2	0.3	21	2.6	23	2.8
	814	0.01	EC_{01}	1	0.1	6	0.7	7	0.9
Rotenone	860	0.05	EC_{10}	91	10.6	117	13.6	208	24.2
	860	0.01	EC_{10}	39		68	8.4	107	12.9
DNOC	703	0.05	EC_{10}	20	2.8	56	8.0	76	10.8
	703	0.01	EC_{10}	5	0.7	17	2.4	22	3.1
	703	0.05	EC_{40}	12	1.7	52	7.4	64	9.1
	703	0.01	EC_{40}	1	0.1	19	2.7	20	2.8

Data obtained from the spot-to-spot analysis for all investigated toxicants are shown in Figure 5-7 and summarised in Table 5-3. For all toxicants and all exposed concentrations significantly overexpressed and repressed proteins were obtained. Except for the DNOC EC10 sample, the number of overexpressed proteins exceeded the number of repressed proteins. Overall, Rotenone, with 24.2 % of all detected proteins, caused the highest percent of significantly changed proteins in the larval proteome, followed by EC_{10} and EC_{40} of DNOC with 20.9 % and 9.1 %, respectively and the EC_{10} and EC_{01} of Diclofenac with 6.8 % and 2.8 %, respectively.

This order is not changed if only the high-significantly more reliably changed proteins ($p < 0.01$) are considered. These are indicated in Figure 5-7 and Table 5-3 as well. Treatment of larvae with the EC_{10} of Rotenone leads to 12.9 % of high-significantly changed proteins related to all detected proteins, followed by EC_{10} and EC_{40} of DNOC with 5.4 % and 2.8 %, and EC_{10} and EC_{01} of Diclofenac with 2.3 % and 0.9 %. The results from univariate spot-to-spot analysis mirror the results from the multivariate analysis showing a sharp separation between control and treatment protein pattern for Rotenone, less clear separation of the treatment conditions for DNOC and low distance between control and treatment groups for Diclofenac (Figure 5-6).

Spot-to-Spot Analysis Results for Rotenone

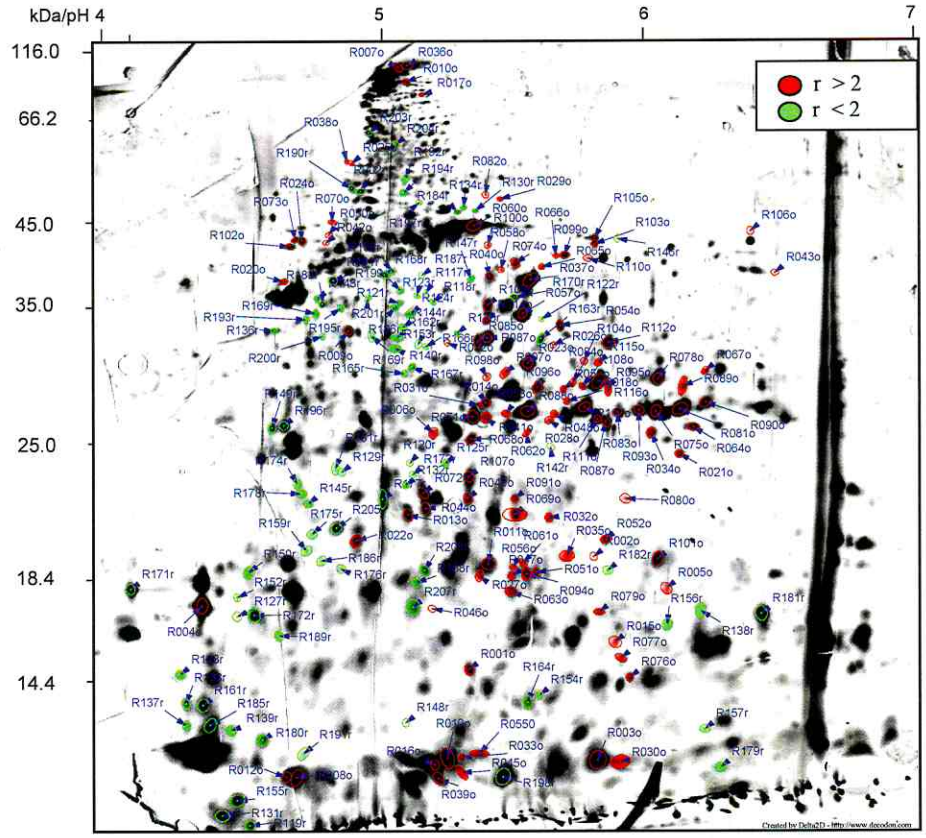


Figure 5-8 Results of univariate spot-to-spot analysis of a toxicoproteomics experiment with ***Rotenone*** (EC_{10}) exposed larvae. The 2-DE experiment comprised three replicates from control larvae and three replicates from Rotenone exposed larvae. The figure presents a fusion gel from all gels in the experiment. 860 detected protein spots were included in the analysis set. All significantly differentially expressed proteins are indicated and labelled on the gel. The labels describe with R (Rotenone) the exposed toxicant, the spot ID (number behind R) and also contents information whether the proteins were expressed at higher levels (red circles, r > 2) or at lower level (green circles, r < 2) compared to controls. From the 860 detected proteins 91 were overexpressed and 116 were repressed.

In detail, for Rotenone 860 detected proteins on the gels were included in the spot-to-spot analysis. One concentration (EC_{10} = 0.05 µM) was tested with a toxicoproteomics approach with Rotenone. From the total protein number 91 (10.6 %) were repressed and 117 (13.6 %) proteins were overexpressed compared to the controls. The differentially expressed proteins showed a pattern distribution on the gels. Whereas most of the repressed proteins appeared at areas with lower pH (pH 4 to 5) on the gels, overexpressed proteins could be mostly found on areas with higher pH on the gels (pH 5 to 6). All differentially expressed proteins are shown on a 2-DE gel in Figure 5-8. The

spot IDs, relative volumes, standard distributions and t-test results for all differentially expressed proteins are summarised in Table B (Appendix).

Spot-to-Spot Analysis Results for DNOC

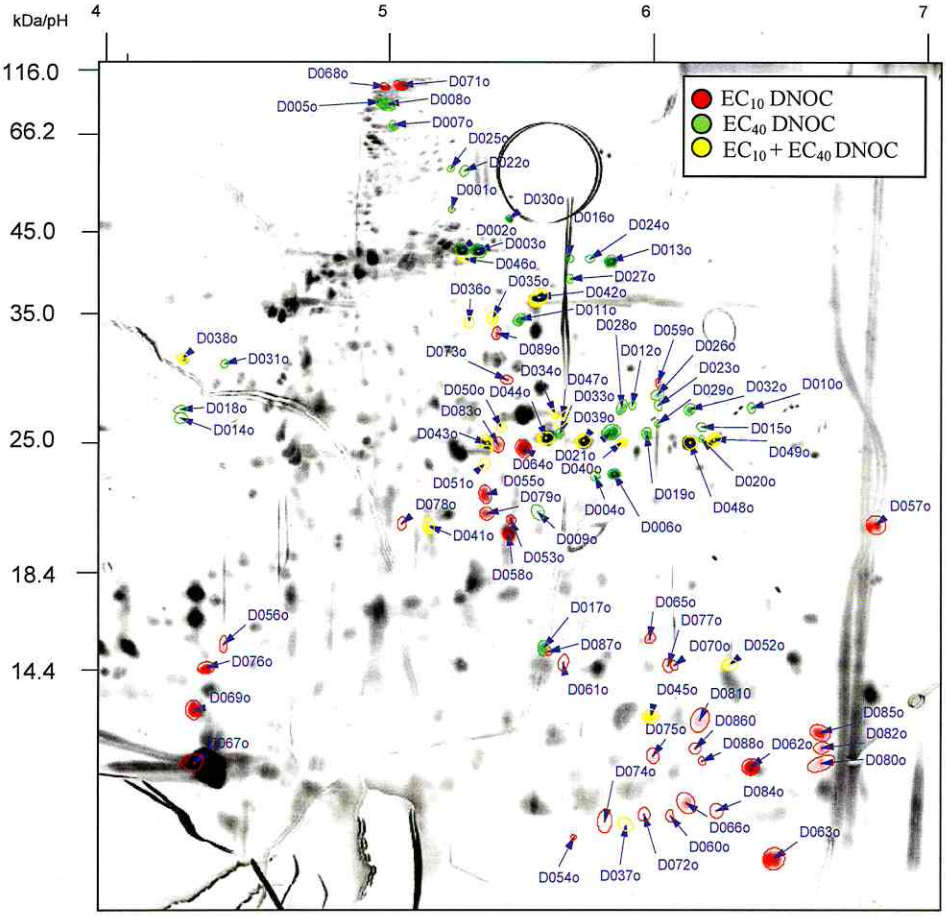


Figure 5-9 The depicted gel is a fused gel from all gels in a 2-DE experiment with two concentrations of ***DNOC*** (D) (EC_{10} and EC_{40}). Altogether, 703 spots were included in the analysis set. On the presented gel only proteins with ***higher expression levels*** (o) compared to controls are marked with colours and labels (D-DNOC, number- spot ID, o-overexpressed,). Red spots are proteins (proteins) with changed abundances only at EC10 DNOC treatment, green marked proteins (52 proteins) show significant changes in expression only after EC40 DNOC treatment. All yellow marked spots showed concurrently higher expression levels compared to controls at both exposure (EC10 and EC40) conditions.

For the toxicoproteomics experiment with DNOC, 703 detected proteins were included in the analysis set. Overall, after exposure with the EC_{10} concentration (2.8 µM) 20 (2.8 %) of all proteins decreased in abundance compared to controls and 56 (8.0 %) of the proteins showed higher expression levels. Interestingly, the treatment of larvae with a higher concentration of DNOC (EC_{40}= 3.5 µM) caused changes in a lower number of

proteins compared to EC_{10}. At EC_{40} 12 (1.7 %) of all proteins were expressed at lower levels and 52 (7.4 %) of the proteins were expressed at higher levels compared to control gels. Like for Rotenone, repressed proteins mainly possessed lower pI values and overexpressed proteins appeared at higher pH values on the gels (Figure 5-9 and 5-10). All overexpressed proteins are indicated in the 2-DE gel in Figure 5-9 and all repressed proteins are shown in the 2-DE gel in Figure 5-10.

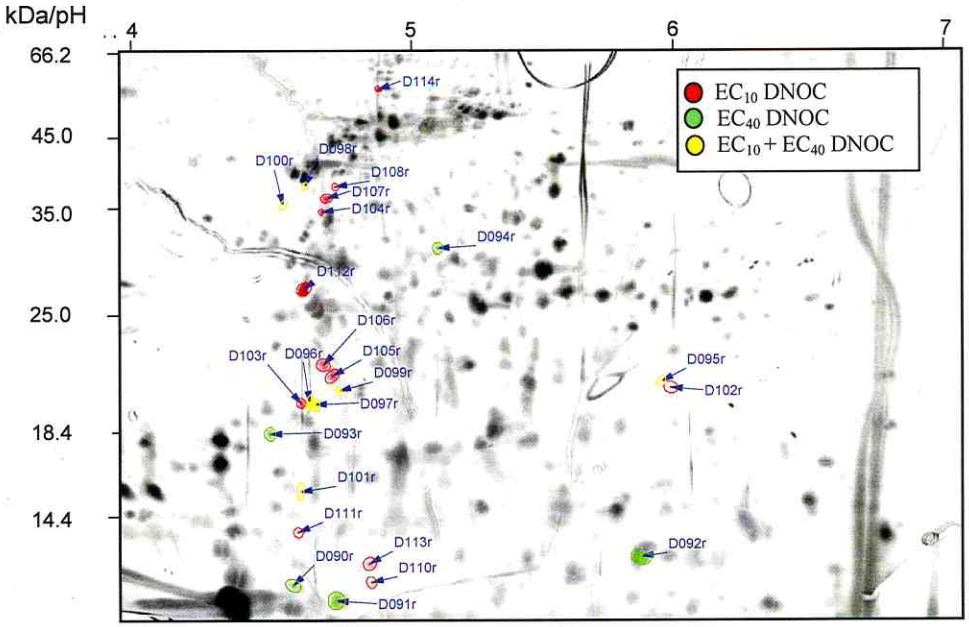


Figure 5-10 The depicted gel is a fused gel from all gels in a 2-DE experiment with two concentrations of ***DNOC*** (D) (EC_{10} and EC_{40}). Altogether, 703 spots were included in the analysis set. On the gel only proteins with ***lower expression levels*** (r) compared to controls are shown and marked with colours and labels (D-DNOC, r-repressed, number- spot ID). Red colour coding: proteins with changed abundances at EC_{10} but not EC_{40} DNOC treatment, green colour coding: proteins showing significant changes in expression at EC_{40} DNOC but not EC_{10} treatment. All yellow marked spots showed significantly decreased expression levels compared to controls at both exposure (EC_{10} and EC_{40}) conditions.

The spot IDs, relative volumes, standard distributions and t-test results for all differentially expressed proteins are summarised in Table C (Appendix). 26 (19 overexpressed and 7 repressed proteins) proteins showed changed expression levels after both EC_{10} and EC_{40} treatment. These are about 40 % of all significantly changed proteins after EC_{40} treatment and about 34 % of all significantly changed proteins after EC_{10} treatment. The proteins having concurrently changed after treatment with both concentrations are marked by yellow colour in the depicted gels (Figure 5-9 and Figure 5-10). However, only eight of the 26 proteins (D36o, D39o, D46o, D48o, D96r, D97r, D99r, D100r) with changed amounts after both treatment conditions showed concentration-dependent trends considering their quantification data (Table C Appendix).

Concentration dependence hereby was referred only to proteins showing trends in their expression change proportional to toxicant concentration.

Spot-to-spot Analysis Results for Diclofenac

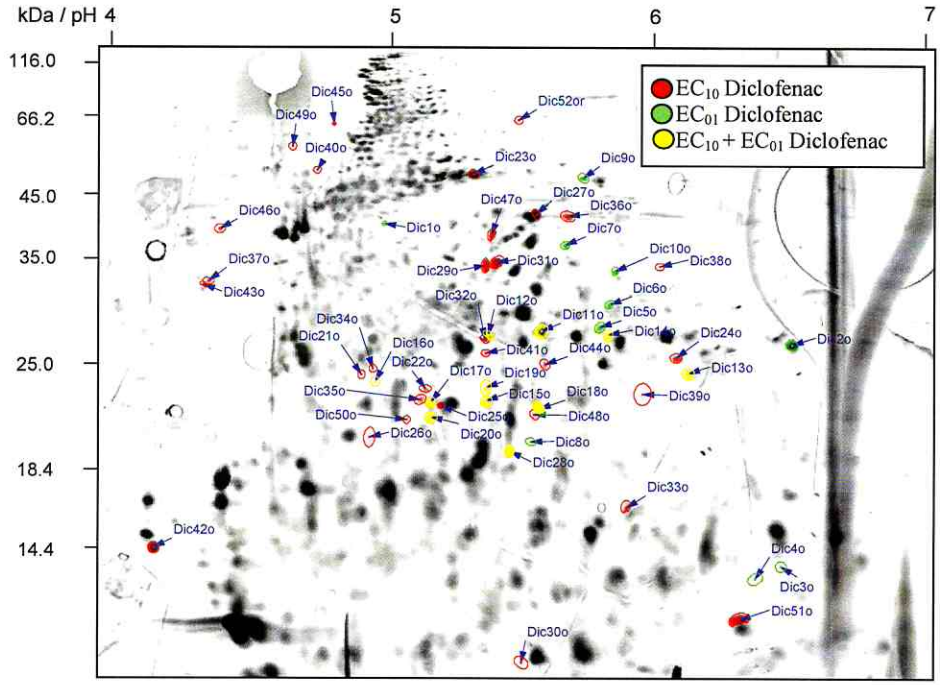


Figure 5-11 Fused gel of all 2-DE gels of a toxicoproteomics experiment with ***Diclofenac*** (Dic). Altogether, 814 proteins were detected on the gels. All ***overexpressed*** (o) proteins in the treated samples are marked and labelled on the gel. (Dic- Diclofenac, number-spot ID, o-overexpression). Red coloured spots are proteins with changed abundances at EC10 but not EC1 Diclofenac treatment, green marked proteins show significant changes in expression at EC1 but not EC10 Diclofenac treatment. All yellow marked spots concurrently showed higher expression levels compared to controls at both exposure (EC1 and EC10) conditions.

Altogether, 814 proteins were included in the analysis set of the toxicoproteomics experiment with two concentrations (EC_{10} = 12.6 µM and EC_{01} = 6.3 µM) of Diclofenac. After treatment with 12.6 µM Diclofenac, 14 (1.7 %) proteins were expressed at lower levels and 41 (5.0 %) at higher levels than in controls. Treatment of the larvae with 6.3 µM Diclofenac caused a change in expression of a lower number of proteins compared to the 12.6 µM treatment. At this concentration, 2 (0.3 %) proteins were significantly reduced and 21 (2.6 %) proteins were significantly increased in abundance compared to controls. Results from spot-to-spot analysis for Diclofenac are depicted in Figure 5-11 and 5-12. The spot IDs, relative volumes, standard distributions and t-test

results for all differentially expressed proteins after EC_{10} or EC_{01} Diclofenac treatment are summarised in Table D (Appendix).

In contrast to Rotenone and DNOC, no clear pattern in the pIs of the overexpressed and repressed proteins could be observed. 11 proteins (around 50 %) with changed abundances at EC1 also showed similarly changed expression at EC10 (marked in Figures 5-11 and 5-12), but only 6 of them (dic13o, dic15o, dic16o, dic17o, dic18o, dic20o) showed clear concentration dependent trends in their fold change ratios in relation to controls (Table D Appendix).

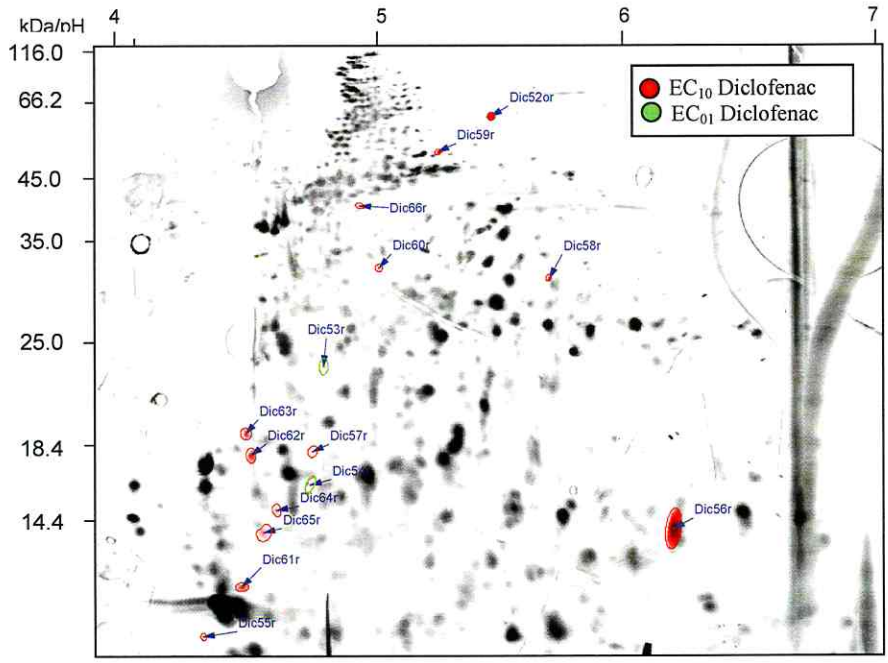


Figure 5-12 Fusion gel of all 2-DE gels of a toxicoproteomics experiment with ***Diclofenac*** (Dic). Altogether, 814 proteins were detected on the gels. All proteins that were ***repressed*** (r) in the treated samples compared to controls are marked and labelled on the gel. (Dic-Diclofenac, number-spot ID, r-repressed). Red marked spots are proteins with changed abundances at EC10 Diclofenac treatment, green marked proteins show significant changes in expression at EC1 Diclofenac treatment.

To summarise, spot-to-spot analysis enabled the identification of several significantly changed proteins, and hence, protein expression signatures (PES) for all analysed treatment conditions. Rotenone treatment caused changed expression levels of the highest number of proteins in the larval proteome, followed by DNOC and Diclofenac. For the two tested concentrations of DNOC and Diclofenac an overlap in proteins showing changed expression levels could be obtained. However, not all of these concurrently changed proteins showed clear dose dependent trends in their fold change

ratios in relation to controls. Considering the number of changed proteins, dose dependent reactions at the proteome level were detectable for the two tested Diclofenac concentrations. In contrast, more proteins were found to be significantly changed for a lower DNOC concentration than for the higher tested DNOC concentration.

Comparison of Spot-to-Spot Analysis Results from Rotenone, DNOC and Diclofenac

Based on the results from spot-to-spot analysis, changes in the larval proteome pattern received from all treatment conditions (Rotenone, DNOC and Diclofenac) were compared to distinguish more generally affected proteins from toxicant specific reactions. Due to the observed biological variability (Figure 5-2), analyses of the toxicoproteomics experiments with the three measured substances were preformed in three independent 2-DE experiments.

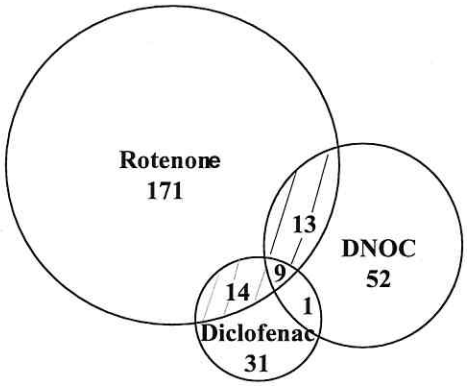


Figure 5-13 Comparison of results from univariate spot-to-spot 2-DE analysis for EC10 exposure against Rotenone, Diclofenac and DNOC. Altogether, 291 proteins were received when the number of all proteins with significant changes in expression levels after treatment of the larvae with the EC10 of Rotenone, DNOC and Diclofenac are added. Most of them changed specific to the toxicants. 9 proteins concurrently changed after Rotenone, DNOC and Diclofenac treatment. Moreover, 13 proteins simultaneously were changed after Rotenone and DNOC treatment and 14 proteins showed changed expression levels at exposure against Diclofenac or Rotenone. Only one protein was found to be changed in the larval protein samples after DNOC as well as Diclofenac treatment.

Hence, to enable the comparison of all experiments, fusion gels from each 2-DE experiment (Rotenone, DNOC and Diclofenac) with all detected significantly changed proteins were matched and the differentially expressed proteins were compared. The results of the overall comparison of the reactions to the three toxicants in the larval proteome are summarised in Figure 5-13 and Table E (Appendix). Only changes in the proteome pattern caused by EC10 concentrations of Rotenone, DNOC and Diclofenac were used for the comparison.

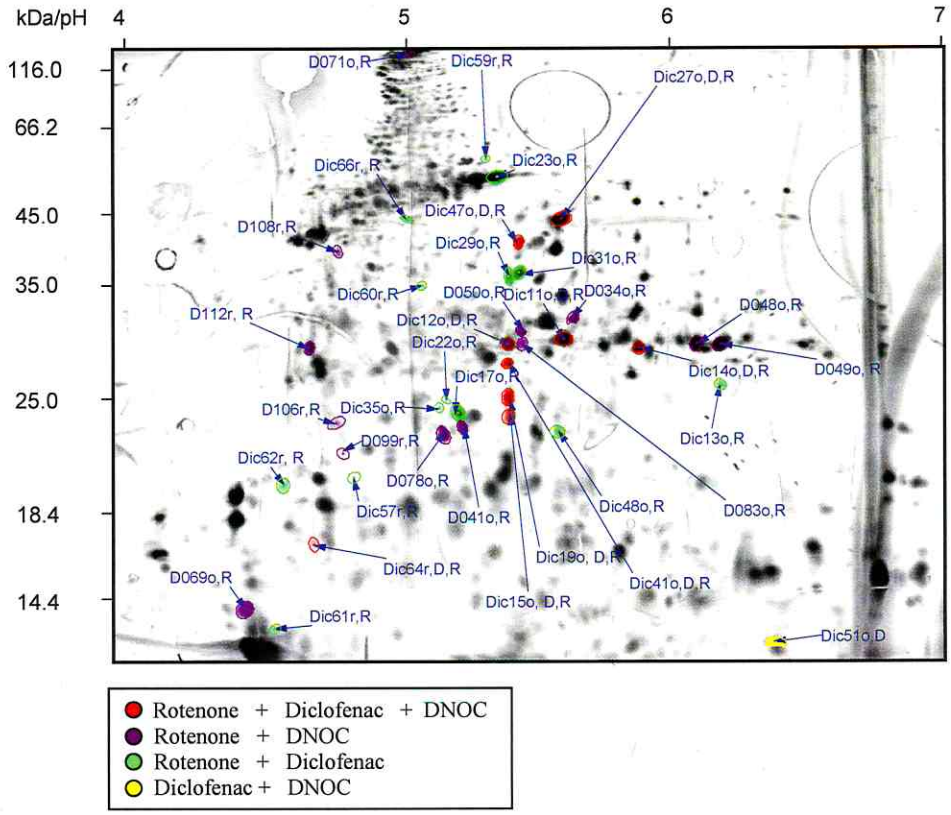


Figure 5-14 On the presented 2-DE gel, all proteins are indicated which were significantly changed in expression levels after treatment with at least two toxicants (EC_{10}): red spots: proteins concurrently changed after treatment with Rotenone, DNOC or Diclofenac (9 spots), purple spots: proteins with changed abundances after Rotenone as well as DNOC exposure (13 spots), green spots: proteins with changed abundances after Rotenone and Diclofenac treatment (14 proteins) and yellow: protein that has simultaneously changed when Diclofenac and DNOC treatments are compared.

To summarise, each toxicant caused its own set of differentially expressed proteins. However, next to substance specific changed proteins also proteins showing changed expression levels after toxic exposure against two or three substances could be obtained.

5.3 Discussion

The aim of the study described in this chapter was to establish proteomics with larvae of the zebrafish and to characterise its potential for toxicoproteomics applications in ecotoxicology to find new toxicity targets, novel biochemical biomarkers indicating toxicant exposure and obtain new insights in molecular mechanisms of toxicity (Monsinjon and Knigge 2007, Dowling and Sheehan 2006). Hereby, the main questions to be answered with this study were whether toxic stress can be detected in the proteome

of treated larvae, whether exposure with different toxicants can be discriminated at the molecular level and whether possible candidate protein biomarkers can be proposed indicating exposure against toxicants. To give answers to these questions, toxicoproteomics approaches using zebrafish larvae were performed with three different compounds, two insecticides, affecting primary metabolic pathways (oxidative phosphorylation), Rotenone and DNOC (Locker *et al.* 1950, Terranova *et al.* 1965), and the pharmaceutical Diclofenac, with *Mode of Action* as anti-inflammatory agent (Vane and Botting 1998).

The results will be discussed in two main parts. Firstly, some methodological aspects including larval age, the applied toxicant concentrations and the analysis methods for the 2-DE experiments are discussed. The second part of the discussion is related in detail to the results of the toxicoproteomics experiments.

Methodological Aspects

Larvae Age

For two reasons, larvae were sampled at 127 hpf for the toxicoproteomics analyses. On the one hand, the observed shift of the 2-DE protein patterns from yolk proteins towards cellular proteins during embryonal development (see Chapter 3) is nearly completed at this developmental stage and Vtg rich areas on the gels are not anymore detectable. Most of the yolk proteins seem to be utilised and are not as high abundant as in younger developmental stages. On the other hand, 127 hpf old larvae are vital and do not show starvation which could be assumed if yolk is nearly utilised and no external feeding of the organisms takes place. However, the proteomes of 127 hpf old larvae obtained from different spawning events could be distinguished by multivariate analyses. It can be speculated that these variances in the proteome pattern result from variances in yolk protein consumption. To our knowledge, the time point of complete yolk consumption in *Danio rerio* has not been investigated so far. However, yolk utilisation may strongly depend on many parameters including movements of the larvae, temperature, light conditions or time point of fish egg incubation. These parameters might slightly vary for each biological sample deriving from larvae and could lead to differences in the proteome pattern. Hence, for all performed toxicoproteomics experiments controls from the same spawning event as the treated samples were included. This is also proposed for any future applications of toxicoproteomics with zebrafish larvae. Next to toxicoproteomics concerns, the exact time point of complete

yolk consumption might be established as an exact and sensitive indicator of development velocity and energy need.

Tested Concentrations

The output of a toxicoproteomics experiment strongly depends on the applied toxicant concentrations and exposure times and should be interpreted related to observed effects at higher biological organisation levels in the analysed organisms. All effects on higher organisation levels (morphological, physiological, behavioural effects) are preceded by effects at the molecular level (Clements 2000). Toxicoproteomics investigations at toxicant concentrations or exposure times that do not cause any microscopically visible effects in the organisms may lead to the identification of proteins involved in the primary toxicological response or adaptation processes. This could be used for extrapolation of effects at higher organisation levels (Monsinjon and Knigge 2007). However, without relation to effects at the phenotypic level, the selection of concentrations or exposure times causing detectable relevant effects at the proteome level might be difficult.

Toxicoproteomics investigations of affected organisms at higher toxicant concentrations or longer exposure times instead could lead to proteins enabling the exploring of effect-translation processes from cell-to-organism or indicate unspecific disruptions at the molecular level. However, the interpretation of proteome data from investigation of organisms showing morphological or physiological effects is less clear than from non-affected organisms since alterations in the proteome as result or cause from effects at higher organisation levels can hardly be distinguished.

The investigation of low-effect concentrations with a toxicoproteomics approach enables both, the testing of a toxicity relevant concentration and the analyses of molecular effects which precede phenotypic physiological or morphological effects. Therefore, in the present study, the EC_{10} concentrations from all tested substances were selected and investigated with the toxicoproteomics approaches whereby all affected organisms were excluded from the experiments.

The EC_{10} concentrations were extrapolated from modelled concentration-effect relationships which were based on all observed quantifiable effects in the organisms. As the standardised test regime for zebrafish larvae (early life stage test) requires exposure shortly after egg fertilisation and for at least 5 days, effect determinations with larvae were done in accordance to the DarT (DarT, Nagel 2002) in terms of exposure time, test

design and toxic endpoints. However, not all the sublethal toxic endpoints defined for embryos were transferable to larvae. On the one hand, sublethal effects like pigmentation impairments or retardation of development are not very adequate to larvae which are exposed beginning three days post fertilisation. On the other hand, larvae are free swimming and microscopically based analyses of cardiovascular functions (e.g. heart beat) which are consulted for analyses of sublethal effects in embryos, are quite difficult. According to this, hardly sublethal effects were detectable for larvae. This could be one reason for the finding that for all three tested substances, the calculated EC_{50} values for larvae were higher than for embryos since all observable effects in the organisms were included for modelling the concentration-effect-relationships. The different definitions of effects in the organisms of different developmental stages could be the reason for the contradicting findings in literature for differential sensitivities of fish embryos or larvae. For salmonids for example, Finn (2007) has described lower sensitivities for embryos than for larvae. In contrast, higher activity rates of detoxification enzymes can be assumed in older larval stages which would hint to lower sensitivities of larvae compared to embryos. This supporting, Wiegand *et al.* (2000) observed higher activities for soluble and microsomal glutathione S-transferases in larvae compared to younger embryonal stages of zebrafish. The ability of metabolisation and biotransformation of chemicals in larval stages would confirm the results of the modelled concentration-effect-relationships for Rotenone, DNOC and Diclofenac for embryos and larvae. For all three tested substances, detoxification and biotransformation processes in fish have been described (Braunbeck and Völkl 1991 (DNOC); Gingerich *et al.* 1968 (Rotenone), Hong *et al.* 2007 and Schwaiger *et al.* 2004 (Diclofenac)). However, for direct comparison of effect concentrations and also toxicoproteomics results of zebrafish in different developmental stages, there is a need for the definition of comparable sublethal toxic endpoints.

Analysis Method

To characterise the potential of a toxicoproteomics approach with zebrafish larvae to reveal toxicant responses at the molecular level, the proteome of treated larvae in comparison to larvae cultivated under control conditions was analysed. Statistical analyses were carried out on the whole data set to filter out interesting protein spots. So far, no generally used processing and analyses tools are proposed for proteomics data and they are subjects of active research (Meunier *et al.* 2005, Kreil *et al.* 2004). In this study, two statistical approaches were applied for data analysis, based on univariate and on

multivariate methods. The outcome of both approaches in proteomics data analyses has been currently reviewed by Monsinjon and Knigge (2007). The univariate methods were applied to detect changes in the expression of individual proteins. The Student's t-Test is the most frequently used statistical test to identify proteins with statistical changes in expression from one condition to the other (Karp *et al.* 2005, Meunier *et al.* 2005) and was chosen as analysis method for the univariate approach in this study. Hereby, the Type I error cutoff of α = 5 % which is currently used in biology was selected for defining of statistical significance. For reducing the number of false negatives and to avoid a loss of information, no Bonferroni correction was applied which is normally necessary in a multiple-test problem (one test per protein spot detected). This supporting, Storey and Tibshirani (2003) and Chang *et al.* (2004) have declared the family wise error rate as much too conservative for the genomewide studies currently being performed. Next to the significance cutoff, a threshold in fold change of expression rate of the proteins in relation to control conditions was applied to create the final set of proteins (PES) with differential expression in the proteomics studies. Although there is criticism in statistics for a fixed threshold (Wang and Ethier 2004), it is considered as biologically important (Marengo *et al.* 2005, Meunier *et al.* 2005). Proteomics studies from Meunier *et al.* (2005) using the Student`s t-Test for univariate data analysis and the same restrictions for definition of differential expression as applied in this study have resulted in no false positives and thus a false discovery rate of 0 %. Hence, all detected proteins in the present study can be assumed to be truly changed in expression. However, the work from Meunier and coworkers (2005) has also shown that the applied analysis conditions resulted in a quite high number (22.4 %) of false negatives. It can be concluded, that some information in the detected PES could be missing.

Multivariate methods are still not commonly used for proteome analyses (Karp *et al.* 2005). The multivariate method like the applied PCA, utilise all of the proteome data simultaneously to look for patterns in expression change and is mostly used to distinguish pathological (treated) from healthy (control) states without prior knowledge of the nature of the proteins (Monsinjon and Knigge 2007) and by overcoming the problems with false positives or false negatives. Results of this study have shown that PCA analysis of the data from the toxicoproteomics studies have clearly distinguished between the treatment and control conditions and hence confirmed the power of multivariate statistics methods within the field of ecotoxicoproteomics. For the application of multivariate analyses methods in ecotoxicometabolomics, Viant and

coworkers (2006) already proposed a considerable potential in environmental risk assessment. Next to pattern analysis, multivariate methods could also allow the characterisation of single affected biomolecules. This was shown for the application of PCA analysis in several metabolomics studies. Information on single affected metabolites was derived from analyses of the PCA loading plots in relation to the scores plots (Viant *et al.* 2006, Mounet *et al.* 2007). To test the applicability of this approach to the proteome data of the toxicoproteomics experiments with larvae was not content of this work but it clearly confirms the potential of a combined approach of proteomics and multivariate analysis in ecotoxicoproteomics and should be an important research content in the future.

Considering the obtained pattern, univariate and multivariate analyses methods resulted in similar results for the toxicoproteomics experiments with zebrafish larvae. However, the robustness of the obtained detailed PES from both approaches should be investigated in future and can give information on the value of a combination of the two methods for the analysis of data from toxicoproteomics.

Results of the Toxicoproteomics Experiments

One question of the present study was whether toxic stress can be detected in the proteome of treated zebrafish larvae that do not show an observable effect (by microscopical inspection). As discussed above, the low effect but toxicity relevant concentration of EC_{10} was tested for all substances. To give an answer to the above question, all affected individuals were excluded from the proteomics study. By application of multivariate and univariate methods for analysis of the toxicoproteomics data, proteome pattern of control and treated larvae could clearly be distinguished for all tested toxicants (Rotenone, DNOC and Diclofenac). All toxicants caused significant changes in expression levels or abundances of certain proteins. Hence, it can be concluded that the toxicoproteomics approach for all measured toxicants lead to detection of a response at the molecular level in larvae not showing microscopically visible effects. This would confirm the proposed possibility of toxicogenomics approaches (Clements *et al.* 2000, Monsinjon and Knigge 2007) to detect effects at the molecular level prior the occurring of effects at the phenotypic level. Moreover, it supports the potential of detection stress responses at the molecular level which might lead to a deeper understanding of molecular mechanisms of toxicity and the identification of novel biochemical biomarkers (Boelsterli 2007, Monsinjon and Knigge 2007).

Specific Protein Expresssion Signatures (PES)

Each tested toxicant caused its own specific pattern of changed proteins and hence, its own protein expression signature. The term PES was firstly introduced by Bradley and coworkers (Shepard *et al.* 2000a, 2000b) to define a set of proteins differing between contaminant exposure and control. Shrader *et al.* (2003) demonstrated the applicability for PES to distinguish between different exposure scenarios with endocrine disruption in zebrafish embryos. By studying mussels originating from differently polluted field sites, Knigge *et al.* 2004 also described a subset of proteins forming a classifier to distinguish between polluted and unpolluted situations. PES can enable the identification of substance specific biomarker patterns which are considered to provide an overcome of the uncertainties associated with the extraction of single protein markers (Knigge *et al.* 2004, Monsinjon and Knigge 2007). This was also shown in a toxicogenomics study which described specific expression profile pattern in zebrafish embryos enabling the discrimination of exposure against 11 model compounds (Yang *et al.* 2007). Hence, our results, which have identified specific protein patterns for each tested exposure scenario, would support the concept of PES and the idea of extraction of information for certain exposure scenarios only from changed protein patterns without having identified single proteins.

Even if a specific *Mode of Action* is known for all tested substances, the order of potencies derived from the effect-concentration ships based on phenotypic toxic endpoints is in accordance with the order of lipophilicity indicating baseline toxicity (Könemann 1980). Rotenone is the most lipophilic tested toxicant (low K*ow* = 4.1), followed by DNOC (log K*ow* = 2.1) and Diclofenac (log K*ow* = 0.7). Hence, a direct discrimination of specific or unspecific narcotic action of toxicants by analysing the effect-concentration relationships is not possible. In contrast, the specific PES obtained for each toxicant gives hints to specific action of the compounds. Thus, molecular effect analysis could enable the discrimination of specific and unspecific narcotic acting chemicals. However, this hypothesis should be confirmed with the testing of several chemicals which exert toxic effects only by narcosis.

General Stress Markers

Although specific PES were detectable for Rotenone, DNOC and Diclofenac, spot-to-spot analyses revealed 9 proteins that showed concurrently significantly changed expression levels after treatment with the EC_{10} concentration of all three substances.

Hence, these proteins seem to generally response to a wider variety of toxic stress. Heat shock proteins are well known stress proteins showing a reaction to many biological and also abiotic stressors (Iwama *et al.* 1998). Hence, the detected 9 proteins might belong to groups of proteins more generally indicating toxic exposure and could thus provide an origin for the developing of unspecific biomarkers distinguishing between control and exposure scenarios. Interestingly, some of the 9 proteins are localised in an area of the gels where many vitellogenins-derivatives have been identified before (Chapter 3) (MW 30 kDa, pH 5.5 - 6.5). Therefore, some of them might be vitellogenins-derivatives and may indicate retardation in development at the molecular level. Retardation in development can only hardly be followed with microscopy based methods in the applied exposure system. Larvae were exposed within the third day post fertilisation and treatment ended after 48 h in the fifth day post fertilisation. The primary difference between the two stages is the length of the organisms. A very sharp visual differentiation between the stages, like within the first two days of development (Kimmel *et al.* 1995), can thus not be drawn. It can be concluded that, if identification results of the 9 proteins with MS based methods or westernblot analysis would confirm the matching to vitellogenins-derivatives, the established toxicoproteomics approach would allow the molecular identification of developmental retardation in 48 h long exposed 5 dpf old larvae which is not possible with microscopy based approaches.

Next to proteins having changed similarly after treatment with all toxicants, also proteins showing differential expression specific to two of the exposed chemicals were detectable. For Rotenone and DNOC about the same number of simultaneously changed proteins was found as for Rotenone and Diclofenac. In contrast, Diclofenac caused expression changes in only one protein which was also significant changed after DNOC treatment. Proteins that show changes in expression levels after treatment with two or more chemicals but not generally at toxic stress could indicate affected similar pathways and hence *Modes of Action* of the exposed substances. Similar reaction to treatment with DNOC and Rotenone can well be expected. Both substances interfere with the oxidative phosphorylation and ATP synthesis (Locker *et al.* 1950, Terranova *et al.* 1965) causing an increase of glycolysis and a deceleration of the cell division (Mitsuhashi *et al.* 1970). Proteins that are changed in expression after treatment with Rotenone and DNOC could therefore include enzymes from glycolysis. Diclofenac is described as an inhibitor of cyclooxygenases (Vane and Botting 1998). But an action as decoupler in oxidative phosphorylation was found as well (Masubuchi *et al.* 2000). Hence, the similarly

changed proteins after treatment of Rotenone and Dicofenac could be ascribed to the decoupling *Mode of Action* of Diclofenac. However, the lower similarity in the PES of Diclofenac and DNOC is astonishing. Clear conclusions on the similarities in the PES of the different substances might, however, only be possible to draw, if the identification results of the proteins of question are available. To summarise, the observed similarities in the changes of the larval proteome after treatment with the three substances show the potential of the toxicoproteomics approach in zebrafish larvae to detect proteins which could be established as biomarkers relating exposure scenarios directly to certain compounds or *Modes of Action*.

Number of Significantly Changed Proteins

For all three substances the same effect-concentration, which lead to microscopically visible effects in 10 % of the treated organisms (EC_{10}), was tested with the toxicoproteomics approach. At the molecular level, however, in terms of the number of differentially expressed proteins, differences in the effect levels could be detected for all three substances. The EC_{10} concentration of Rotenone caused the change of in total around 25 % of all detected proteins in the larval proteome, the EC_{10} of DNOC of around 11 % and the EC_{10} of Diclofenac of around 7 % of all proteins. Hence, the observed effect level at the molecular level differs from the effect level derived from analysis of more classical toxic endpoints at the phenotypic level.

Interestingly, the number of changed proteins correlated with the determined potencies of the substances. Compared to DNOC and Diclofenac, Rotenone showed the highest potency in larvae with an EC_{50} value of about 68 nM and, in the same time, caused the expression change of the highest number of proteins. Diclofenac in contrast showed the lowest potency with a modelled EC_{50} value of 23.1 µM and also lead to a change of only a small number of proteins. Therefore, by analysing the number of changed proteins, effect analysis at the proteome level, in contrast to classical toxic endpoints, could give hints to the variety of *Modes of Action* of a certain toxicant and information on how basal the affected metabolic pathways are. Rotenone and DNOC have their *Mode of Action* in a primary metabolic pathway, the oxidative phosphorylation (Terranova *et al.* 1965, Locker *et al.* 1950) and caused a higher effect in the larval proteome compared to Diclofenac with the primary *Mode of Action* in a more secondary biochemical pathway by inhibition of cyclooxigenases (Vane and Botting 1998). Impairments in energy metabolism affect many cellular processes and are in direct

relation to changed rates of biosyntheses, like general protein biosynthesis, which cost ATP. Moreover, many enzymes are involved in the general energy metabolism pathways. Hence, changes of many proteins in the larval proteome after Rotenone or DNOC treatment can be well expected. For Rotenone, this was also confirmed by a proteomics study from Jin *et al.* (2007) who identified 110 significantly changed mitochondrial proteins in Rotenone exposed dopameric cell lines.

Rotenone and DNOC exert their major toxic effect in the same metabolic pathway. However, for both substances, differences in the number of proteins with changed expression rates could be detected. The reasons for this might be found in the direct *Mode of Action*. Whereas Rotenone, by inhibition of the oxidative phosphorylation, completely blocks the metabolic pathway (Schuler and Casida 2001), DNOC acts as a decoupler and does not directly inhibit the oxidative phosphorylation. The DNOC effect can be compensated by an increased citric acid circle and glycolysis. The action of Rotenone can only be compensated by an increased anaerobic glycolysis and the shift towards metabolisation of certain biomolecules like lipids. Hence, Rotenone *Mode of Action* might lead to a change in the energy metabolism and thus in protein expression of metabolic enzymes.

Next to oxidative phosphorylation inhibition, multiple other actions are described for Rotenone. These include for example the inhibition of microtubulin formation or the causing as neurodegenerative disorders (Bove *et al.* 2005, Cheng and Farrell 2007, Bretaud *et al.* 2004, Brinkley *et al.* 1974). This is in contrast to DNOC for which beside of acting as decoupler no other *Modes of Action* have been described, so far. This might be also consulted for explanation of the high number of changed proteins after Rotenone treatment compared to DNOC.

Down- and Up-Regulated Proteins

For all three substances more up- than down-regulated proteins were detected. The expression of proteins involved in detoxification mechanisms like glutathione transferases, heat shock proteins, cytochromes, catalase, etc. are known to be induced by toxicants (Boelsterli *et al.* 2007). Reduction of expression levels through toxicants might indicate inhibitions of certain biochemical pathways like protein biosynthesis or hint to general reductions of some biochemical side-pathways to advance the strict concentration on the detoxication processes and the maintenance of the basal metabolic pathways. Hence, it might be speculated, that the higher number of overexpressed proteins indicate

induction of expression of stress proteins like e.g. proteins involved in detoxification or sequestration processes, chaperons or which are related to oxidative stress (Hagger *et al.* 2006, Boelsterli 2007) through Rotenone, DNOC and Diclofenac.

In the following, an additional explanation of this finding is discussed. Next to the high number of over-expressed proteins, for Rotenone and DNOC, also a pattern of over-expressed and repressed proteins was found on the 2-DE gels. Most of the over-expressed proteins possessed higher pI values and many repressed proteins were found on an area of the gel at lower pH values. This pattern could on the one hand be caused by expression induction of proteins possessing generally higher pI values. But on the other hand, the results might be interpreted as a shift of the proteome pattern from exposed larvae towards proteome pattern of younger organisms. Results from Chapter 3 have shown that the proteome of zebrafish embryos is dominated from proteins with higher pI values which were mostly identified as vitellogenins-derivatives. In 72 hpf old larvae (the time point of begin of exposure), the protein pattern still shows many yolk proteins with higher pI. Thus, the general finding of the shift in the proteome pattern of exposed five dpf old larvae towards proteins with higher pI could also be caused by a retarded yolk protein utilisation and indicate a retardation of development at the molecular level. However, shifts in pI values of proteins might also hint to changes in post translational modifications (PTM, like phosphorylation) (Halligan *et al.* 2004) of the proteins. Change of PTM after treatment with toxic chemicals is quite possible because they greatly affect the function of proteins (Halligan *et al.* 2004, Lee *et al.* 2001, Cash *et al.* 2002). However, only identification of the significantly changed proteins would enable to distinguish between these competing interpretations.

Concentration Dependence

Concentration dependence in effects analysis means the manifestation, positive correlation, of the exerted effects with rising concentrations and ensures a direct relation of the measured effects to the toxic exposure. To analyse if concentration-dependent effects can also be observed on the proteome level for different concentrations of Diclofenac and DNOC, next to EC_{10}, the estimated EC_{01} concentration for Diclofenac and the estimated EC_{40} concentration for DNOC were also tested. Dose dependence at the proteome level could include the number of differentially expressed proteins at different toxicant concentrations as well as the quantification data of the single proteins.

Interestingly, for each concentration of the exposed toxicants, specific PES were obtained. Data from Shrader *et al.* (2003) and Meiller and Bradley (2002) also show specific protein pattern for different concentration of the same chemicals and the authors suggested that proteins appear and disappear according to different stress events. This would hint to a possibility to assay the degree of stress with a proteomic approach.

However, proteins having significantly changed at both concentrations of DNOC or Diclofenac could be detected as well. But not all of them showed a dose-dependent trend considering their fold-change ratio to their control counterparts. Nevertheless, all similar regulated proteins at different concentrations seem to be specific for the toxicant and are persistent in the tested concentration range of the substances. Whereas for the two Diclofenac concentrations dose-dependence was observed considering the number of significantly changed proteins, for DNOC the opposite was found: More proteins were differentially expressed at the lower DNOC concentration then at the higher DNOC concentration. This was also observed for a treatment of zebrafish embryos with nonylphenol by Shrader and co-workers (2003). It might be speculated that at higher levels of toxic stress the organism lacks the energy for detoxification processes and all proteins with changed expression levels might therefore indicate disturbance in the proteome.

Identification

Of course, if pattern-only analysis provide some information on the MOA of the toxicants at the molecular level, only MS based identification of all proteins of interest would enable the interpretation and explanation of the obtained data. Identified proteins may give hints to induced detoxification processes of the toxicants, affected metabolic pathways, or the establishing of potential biomarkers. So the identification of proteins specific to toxicants or different exposure scenarios should be the next step in the future. The efforts of identification of the most important proteins have failed so far because of a high contamination of human keratin. This could be caused by contamination during the identification method. Moreover, most of the excised spots were quite small and due to the sensitive silver staining method, detecting of proteins in the nanogram range, keratin contamination could hamper the proper identification of the proteins with lower abundance. A DIGE approach, a fluorescence based sensitive and MS compatible protein visualisation method, could increase the identification success (Gade *et al.* 2003,

Marouga *et al.* 2005). First attempts have been carried out to adapt the established proteomics method for larvae to the DIGE staining technique.

However, the accessibility of the established toxicoproteomics method to protein identification was shown in Chapter 3. In the proteome of untreated larvae, next to several muscular and structural proteins, also one stress associated protein, a heat shock protein, was identified. As many of the identified proteins were high abundant proteins it can be speculated that also the stress dependent changed proteins could belong to protein classes with high expression levels, like house keeping proteins. The approaches for biomarker detection based on muscle proteins (Berna *et al.* 2007, Knowles *et al.* 2004) show the potential of analysing modifications in higher abundant protein classes. The involvement for higher abundant proteins in toxic stress response was also summarised by Monsinjon and Knigge (2007). However, to obtain insights in levels of proteins with lower abundances, the amount of the most abundant proteins in the protein samples should be reduced. Several methods like immobilised dyes or immunoaffinity methods are described for reduction of high abundant proteins (Ahmed and Rice 2005, Righetti *et al.* 2006, Thulasiraman *et al.* 2005).

Summary

Proteomic analysis of protein samples deriving from exposed zebrafish larvae resulted in substance specific PES for Rotenone, DNOC and Diclofenac. Hereby, proteins which showed concentration and substance specific changes in their expression levels as well as proteins showing a more general response to the applied toxic stress were detected. All changes in the proteome were obtained in protein samples from organisms not showing any microscopically visible effects. Hence, toxicoproteomics with zebrafish larvae was established and its high potential to sensitive detect toxic responses at the proteome level was confirmed. By subsequent identification of the proteins with changed expression levels, they could provide the possibility to establish novel biochemical biomarkers and to relate certain biochemical pathways to toxic stress. For this, sensitive and successful identification techniques should be established for the established 2-DE approach with zebrafish larvae. One approach could address the application of the MS compatible fluorescence based DIGE protein visualisation technique.

CHAPTER 6

Cathepsins as a new Family of Biomarkers in Zebrafish Embryos for Normal Embryonal Development and Toxic Exposure

6.1 Introduction

This chapter addresses the establishment of novel biomarkers from the applied ecotoxicoproteomics approaches with zebrafish in early life stages described in the chapters before. The deriving of candidate biomarkers is based on the finding that toxicant exposure induced changes in yolk protein patterns by impairing the high regulated vitellogenins proteolytic processing (Chapter 4). It was observed that proteolysis of vitellogenin-deriving yolk proteins was retarded upon 48 h Ethanol exposure. Vitellogenins (Vtgs) are phosphoglycolipoproteins and as major yolk proteins in all oviparous vertebrates they carry most of the nutrients essential for the developing embryos (Byrne *et al.* 1989, Wallace *et al.* 1969). Vtgs are predominantly produced in the female adults and subsequently taken up into the oocytes (Byrne *et al.* 1989, Tyler and Sumpter 1996, Wallace *et al.* 1969, Wang *et al.* 2005). To supply the embryo with nutrients until it is capable of free feeding, proteolytic processing of the yolk proteins occur during oogenesis and embryogenesis. The enzymatic machinery involved in the yolk protein processing and their regulation during oogenesis and embryogenesis are, however, largely unknown for oviparous species (Fabra and Cerda 2004, Tingaud-Sequeira and Cerda 2007). For many oviparous animals including teleosts, insects and amphibians, the proteases of the cathepsin-type were shown to be implicated in the yolk degradation processes (Carnevali *et al.* 1999, 2001, 2006, Fagotto 1995, Kwon *et al.* 2001, Sire *et al.* 1994, Qiu *et al.* 2005,Yoshizaki and Yonezawa 1994). Cathepsins are intracellular endoproteases that are actually known to be responsible for the general lysosomal protein breakdown. The cystein proteinases cathepsin B and L as well as the aspartic cathepsin appear to function in yolk-formation and -processing of lower vertebrates and birds (Carnevali *et al.* 2006, Hiramatsu *et al.* 2002, Tingaud-Sequeira and Cerda 2007). For the teleost *Fundulus heteroclitus* and the crustacae *Marsupenaeus japonicus*, expression of other cathepsins isoforms like S, K, H, C, Z and F in ovarian follicles have also been described (Fabra and Cerda 2004, Qiu *et al.* 2005). Next to cathepsins two other enzymes, namely lipoprotein lipase (LPL) (*Oncorhynchus mykiss*) (Kwon *et al.* 2001) and tyrosine phosphatase (*Boophilus microplus*) (Silveira *et al.* 2006) were found in eggs. Therefore, yolk degradation seems to be a complex interaction of a whole set of different proteinases.

In contrast to cellular lysosome enzymes, which immediately cleave all proteins down to free amino acids, yolk lysosomal enzymes seem to be controlled through temporal and developing stage depended regulation mechanisms (Carnevali *et al.* 1999,

2001, 2006, Fabra and Cerda *et al.* 2004, Kwon *et al.* 2001, Sire *et al.* 1994). Programmed and timely processing in nutrient release is required to supply the embryo with energy along the whole duration of development. Various regulation processes of the yolk lysosomal system including temporal enzymatic activation of proenzymes or acidification processes in the yolk platelets have been repeatedly studied (Abreu *et al.* 2004, Carnevali *et al.* 2006, Fabra and Cerda *et al.* 2004, Fagotto 1995, Luckenbach *et al.* 2008, Matsubara *et al.* 2003, Raldua *et al.* 2006, Sire *et al.* 1994, Wood *et al.* 2004).

To our knowledge, no data for yolk enzymatic processing in zebrafish are available. However, the expression rate of one cathepsin L gene (ctsla) and the localisation of CatS and CatC mRNA in the yolk syncytical layer of zebrafish embryos were described by Tingaud-Sequeira and Cerda (2007). Data base search in UniProtKB/Swiss-Prot and TrEMBL for cathepsins reveal five cysteine proteinases [cathepsin L –like proteases (CatL, K and H), cathepsin B and cathepsin C (dipeptidyl peptidase 1)] as well as one aspartic cathepsin CatD. Their activity rates and roles in embryogenesis have not been studied yet.

As we could demonstrate impaired yolk protein patterns due to toxic stress in *Danio rerio* embryos, this chapter aimed to test the hypothesis that activities of cathepsins could be useful novel biomarkers indicating abnormal yolk degradation and hence impairments in embryonal development. They may serve as additional endpoints in zebrafish embryo toxicity protocols. Therefore, the activities of three cysteine proteases CatH, CatC and CatL and their reaction to four reference substances, namely Leupeptin, a cathepsins L inhibitor, Ethanol which caused changes in the yolk protein pattern of 2 dpf old embryos (Chapter 4), DNOC and Rotenone, with *Mode of Action* mainly in the energy metabolism, were analysed in zebrafish embryos in this study.

6.2 Results

6.2.1 Cathepsin Activities in Embryonal and Larval Stages of the Zebrafish

Preceding the toxicity related analysis, cathepsin activities during *Danio rerio* development at eight different embryonal stages starting shortly after egg fertilisation (0.5 hpf) to free feeding larvae (126 hpf) were studied. Hereby, we characterised CatC, CatH and CatL substrate conversion rates in the fish embryo samples. For CatL and CatH substrates, beside specific enzymatic conversion (CatL and CatH activity) also unspecific conversion processes were observed in the egg samples. Hence, for these substrates next

to specific also unspecific cleavage rates and as sum of both, total conversion rates were analysed. No unspecific conversion for CatC substrate was assumed because CatC (dipeptidyl aminopeptidase I) substrate is described to be very specificly converted by dipeptidyl aminopeptidase I (Chan *et al.* 1985).

To enable an exact comparison of enzymatic activities (in units) from different protein samples, they are normalised to the total protein amount in the samples and termed as specific activities (units per mg protein). It should be noticed that in the present work the term of specific substrate conversion is related to the specificity of the enzyme and is different from the term of specific activity which is defined as the activity of the enzyme related to the total protein amount in the sample.

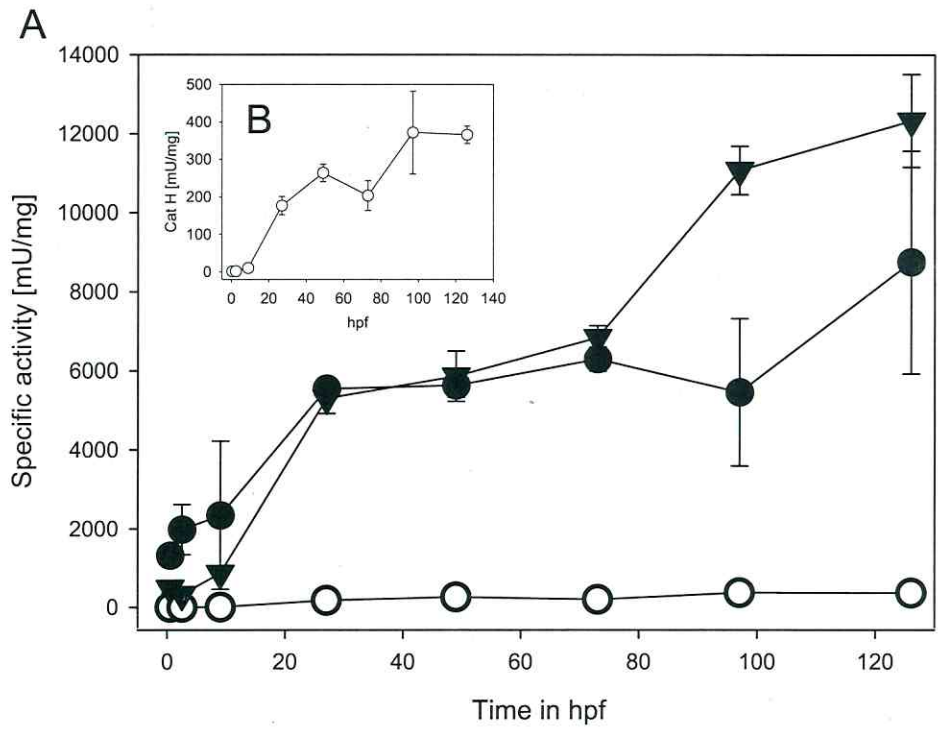


Figure 6-1 A) Specific activities, related to protein concentration in the samples, of specific conversion of CatL (▼), CatC (●) and CatH (○) substrates for eight different embryonal stages are depicted. **B)** Enlargement of the figure of specific activity of CatH.

As a first step towards characterisation of cathepsins in embryonal samples, the specific activities of the specific conversion of the three cathepsins substrates were investigated for different developmental stages. Interestingly, all measured specific activities for CatL, CatC and CatH continuously increased from 0 to 5 dpf (Figure 6-1).

This could be due to a constant reduction of the amount of yolk proteins and therefore the total protein content per egg during the timeframe of development as was investigated before (see Chapter 3, Figure 3-7). Hence, the cathepsin activity values could be misinterpreted when data are normalised to the total protein amount in the samples. Therefore, for all subsequent comparisons of cathepsins activities of different developmental stages, absolute activity values were consulted and no normalisation step in relation to the total protein amount in the samples was performed. To ensure comparability of enzyme activities from different samples, normalisation was realised by a constant relation of lysis buffer to number of pooled embryos.

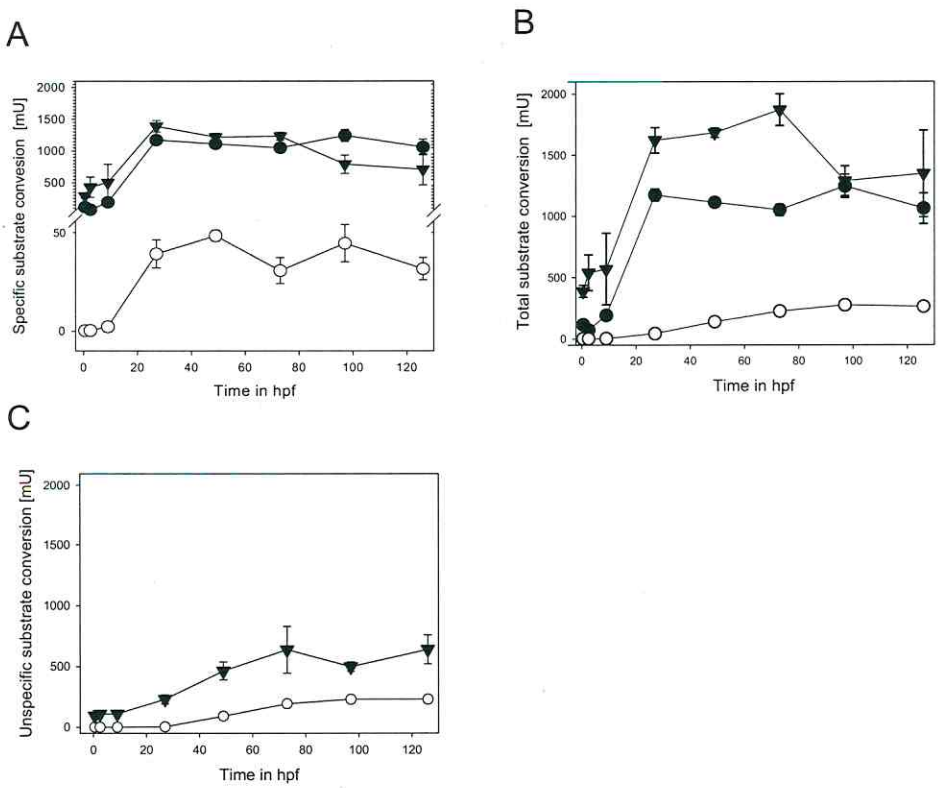


Figure 6-2 Cathepsin activities during embryonal development. Activities were characterized at eight different time points (0.5, 2.5, 9, 27, 49, 73, 97 and 126 hpf). CatL (▼), CatC (●) and CatH (○) substrate conversion were analysed after homogenisation of pooled embryos. Experiments were done in triplicates and standard deviation is indicated for each measured data point. Unspecific and specific substrate conversions were distinguished by the use of specific Cat inhibitors. No unspecific substrate conversion for CatC was observed. (**A**) shows specific substrate conversion, (**B**) shows the total substrate conversion and (**C**) depicts the unspecific substrate conversion.

Overall, for all measured substrate conversion processes (total, specific and unspecific) only minor enzymatic activities were detectable in the early stages from 0.5 to 9 hpf (90 % epiboly). A strong increase (at least 10 fold) in activities occurred between 9 and 26 hpf (prim 6 stage) (Figure 6-2).

Whereas CatC and H activities (specific CatC and H substrate conversion) subsequently remained constant, CatL activity (specific CatL substrate conversion) showed the highest peak at 26 hpf and slightly decreased until 5 dpf (Figure 6-2 A).

The total CatL substrate conversion rate reached its highest rate at 3 dpf and also showed a decline until 127 hpf (Figure 6-2 B). In contrast, the total CatH substrate conversion and also all measured unspecific activities more or less constantly increased during the embryonal development (Figure 6-2 C). However, almost all measured activities (total, specific and unspecific activities) showed a decrease from 4 to 5 dpf. With values above 1 U, CatL and CatC showed around 30 fold higher activities than CatH at all developmental stages. This is also true if total CatL and CatH substrate conversion rates are investigated (Figure 6-2 B). Except for older larval stages, CatL exerted the predominant enzymatic activity.

6.2.2 Leupeptin as Positive Control for direct *in vivo* Cathepsin Inhibition

To analyse if direct cathepsin inhibition can be detected in the established *in vivo* system, embryos were exposed to the CatL inhibitor Leupeptin. Morphological effects of Leupeptin were determined using the DarT (Schulte and Nagel 1994). Exposure time was 48 h and effects were analysed in 2 dpf old embryos (Figure 6-3).

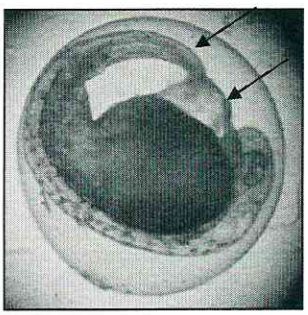


Figure 6-3 52 hpf old embryo exposed to 0.3 mM Leupeptin for 48 h. The embryo shows sublethal effects like oedema in the heart and yolk region, an affected blood circulation and a distorted backbone as indicated by the arrows.

Leupeptin caused sublethal effects, like affected blood circulation, oedema in the heart region and retarded development at lower concentrations and induced lethal effects like coagulation at higher concentrations. Figure 6-4 shows the modelled concentration effect relationships for either sublethal or lethal effects.

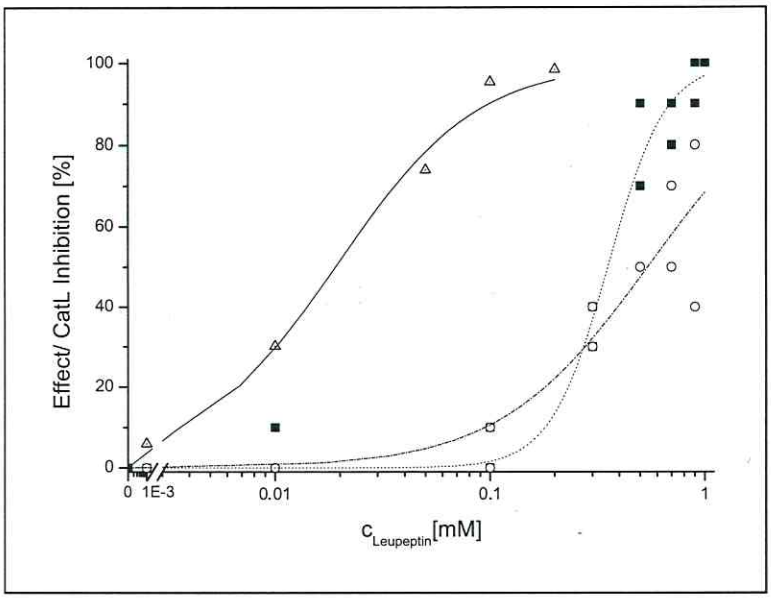


Figure 6-4 Effect characterisation in 50 hpf *Danio rerio* embryos after 48 h Leupeptin (CatL inhibitor) exposure. Concentration effect relationships for the DarT assessment of lethal effects (○) [LC_{50} = 0.542±0.068, p = 260±0.30851, R^2 = 0.866] and sublethal effects (■) [EC_{50} = 0.35±0.019, p = 3.269±0.515, R^2 = 0.969] in *Danio rerio* embryos and CatL activity inhibition (Δ) [IC_{50} = 0.019±0.002, p = 1.334±0.175, R^2 = 0.98966] measured in homogenised embryos are shown. Observed lethal effects were coagulation, no heart beat and no blood circulation and as sublethal effects, except of pigmentation impairments, all detected effects, like oedema or distortion in the backbone, were included.

The calculated EC_{50} and LC_{50} values are 0.35 mM and 0.54 mM, respectively. Four concentrations between 0.001 and 0.2 mM were chosen to analyse effects of Leupeptin to the CatL activity. To exclude Leupeptin contamination in the assay, after exposure, embryos were intensively washed with *aqua dest.* before freezing and protein isolation. For all chosen concentrations decreased activities in CatL compared to controls could be detected. The resulting concentration effect relationship is shown in Figure 6-4 and the determined IC_{50} value is 0.019 mM. This is about 18 times lower than the EC_{50} determined from DarT and even 28 times lower than the LC_{50}. About 95 % inhibition of CatL was already seen at 0.1 mM Leupeptin where only around 10 % of the embryos showed microscopically detectable effects. For this concentration also strong effects on a 1D proteome pattern of whole embryos could be detected (Figure 6-5). Compared to protein patterns of younger embryos, protein bands at 118 kDa and 66 kDa (Figure 3-3 line 4, Chapter 3) hint to retardation in yolk degradation after Leupeptin exposure.

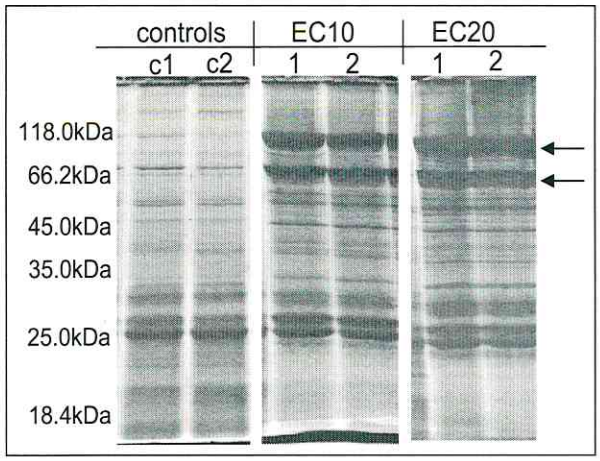


Figure 6-5 1D SDS-PAGE of protein samples from control and Leupeptin treated embryos. For each exposure condition [control, EC10 (0.1 mM) and EC20 (0.2 mM) of Leupeptin] two replicates are shown. Exposed and control protein pattern mainly differ by 2 protein bands with higher molecular weight (118 kDa and 66 kDa). The shown arrows indicate proteins bands which are supposed to be vitellogenin derivatives. 30 µg of protein was loaded on each line.

6.2.3 Effect of three Chemicals on the *in vivo* Activity of the Cathepsins C, H and L

Three reference substances Ethanol, Rotenone and DNOC were chosen to investigate effects on the *Danio rerio* cathepsin activities as potential biomarkers for toxic exposure. Embryos were exposed for 48 h to the toxicants and enzymatic activities were determined in 2 dpf old embryos. Modelled EC_{20} values (Ethanol: 0.7 % (v/v), DNOC: 0.27 mg/L, Rotenone: 8.1 µg/L) were used as exposure concentrations for all chemicals. The EC_{20} values were deduced from concentration effect relationships based on sublethal effects analysed by DarT which are summarised in Figure 6-6 for DNOC, Rotenone and Ethanol. The original data for Ethanol are taken from Chapter 4 and for DNOC and Rotenone from Chapter 5. Only embryos showing no microscopically visible effects were included in the enzymatic measurements.

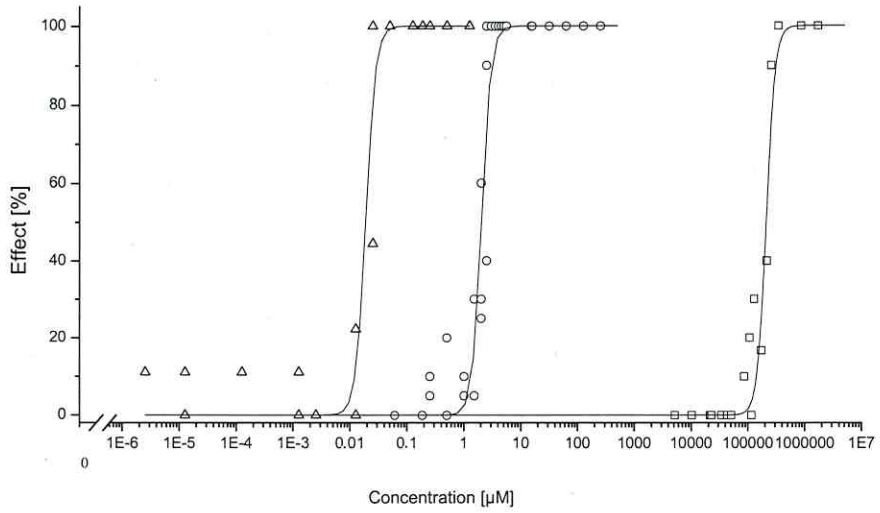


Figure 6-6 Modelled concentration effect relationships from 48 h treatment of larvae with Rotenone (Δ), DNOC (○) and Ethanol (□). Data and parameter estimates were taken from Chapter 5 (Figure 5-5 for Rotenone and DNOC) and from Chapter 4 (Figure 4-4 for Ethanol). The presented concentration relationships are derived from observed sublethal and lethal effects.

Cathepsin activities in *Danio rerio* embryos under control or exposed conditions are shown in Figure 6-7. Data of specific substrate conversion for CatC, CatH and CatL (Figure 6-7 A, B, C) substrates as well as total and unspecific substrate conversion for CatH and L substrates (Figure 6-7 B and 6-7 C) are presented. Each data point is derived from 540 *Danio rerio* embryos (n = 9, with 60 embryos each). Since all embryos were from the same developmental stage (2 dpf) specific activities, normalised to mg of total protein amount, were taken for analyses.

For Ethanol, no significant changes in specific Cathepsin C, H and L substrate conversion processes were observable in the exposed embryos (Figure 6-7 A, B, C). However, a trend towards increased specific CatC activity can be assumed ($p = 0.14$) (Figure 6-7 A) and was confirmed by significant changes in CatC ($p < 0.0001$) activities after Ethanol exposure in the activity data not normalised to protein amount.

In contrast to Ethanol, Rotenone exposed embryos showed significantly decreased activity rates for all measured enzymatic activities (Figure 6-7 A, B, C). Hereby, total, specific as well as unspecific CatC, CatH and CatL substrate conversion processes were reduced compared to controls. Strongest inhibition effects of Rotenone could be detected for total and unspecific CatH substrate conversion processes (Figure 6-7 B).

As for Rotenone, also DNOC lead to highly significantly reduced total and unspecific CatH processes (Figure 6-7 B) and also decreased unspecific CatL substrate conversion. But no effects towards specific CatH activity, CatC activity or total and specific CatL substrate conversion were detectable for DNOC (Figure 6-7 A, B, C).

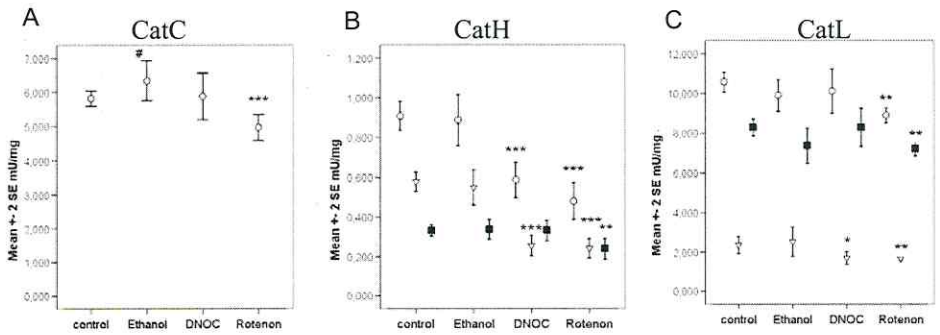


Figure 6-7 Effects of EC_{20} concentrations of Ethanol (0.7 % v/v), DNOC (0.27 mg/L) and Rotenone (8.1 µg/L) to the specific activities of cathepsins C, H and L [mU/mg protein] are depicted. All shown data points derive from nine replicates with 60 homogenised embryos each. The mean values plus/minus the standard error (SE) are shown. Values are presented compared to controls. For CatH (**B**) and CatL (**C**) next to total substrate conversion (○), also specific (■) and unspecific (Δ) substrate conversion is shown. As for CatC (**A**) no unspecific enzyme reactions were observed, only the total (= specific) activity is shown. Statistically significant differences to the control are indicated by asterisks (* $P < 0.05$,** $P < 0.01$,*** $P < 0.001$). The # symbol indicates observed statistically significant differences to the control considering the activity values (mU) when data are not normalised to protein concentration.

6.2.4 Concentration Dependence of Effects on Cathepsin Activity

To validate the effect data received from exposure with EC_{20} of the substances (Figure 6-7) and to get information about dose dependence of the observed effects, concentration series of the three substances were characterised. In Figure 6-8 (A, B, C) the concentration-effect-relationships for conversion rates of different cathepsin substrates at different concentrations of DNOC, Ethanol and Rotenone are depicted. Like for the tested EC_{20} values, only intact organisms showing no morphological effects, were included in the enzymatic measurements. Hence, to ensure the required number of intact organisms, no toxicant concentrations exceeding the EC_{80} were tested with enzymatic approach. Effects to cathepsin activities are shown in relation to the 'reciprocal' effect-relationships (no-effect curves) for lethal and sublethal effects deriving from the DarT concentration effect relationships shown in Figure 6-6.

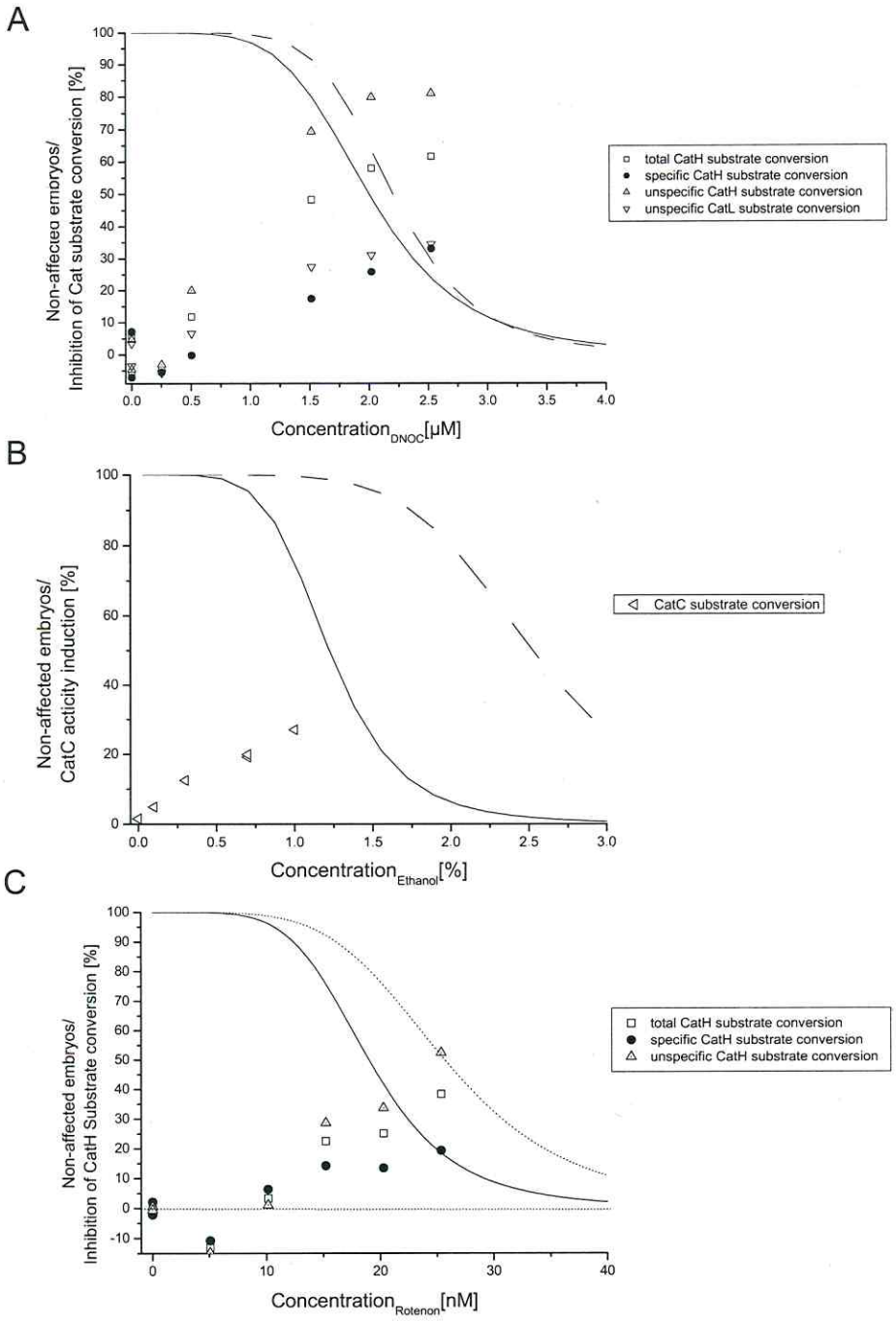


Figure 6-8 Concentration dependent effects of DNOC (**A**), Ethanol (**B**) and Rotenone (**C**) to cathepsins activities in comparison to the reciprocal concentration-effect curves (DarT) for investigated sublethal and lethal effects are depicted. Only cathepsin activities showing concentration dependent effects are presented. Reciprocal concentration-effect-curves for sublethal effects (—) or lethal effects (---) deriving from DarT are depicted in the same diagrams.

Concentration dependent effects in intact organisms on Cathepsin substrate conversion rates were found for all tested chemicals. Moreover, clear effects already appeared at concentrations not leading to any lethal or, for Ethanol and DNOC, to sublethal effects. Almost all effects which were observed after EC_{20} of the tested chemicals could be recovered as being also concentration-dependent. However, effects previously seen for CatL and CatC after Rotenone exposure (Figure 6-7 A, C) did not show clear concentration dependence and are not depicted in Figure 6-8.

With values up to 80 % for the inhibition of unspecific CatH cleaving enzymes, DNOC lead to the highest detected efficiency of the cathepsin activity inhibition effects (Figure 6-8 A). At this concentration (2.5 µM DNOC) also quite high inhibition rates for specific (~ 30 %) and hence total CatH substrate conversion (~ 60 %) and unspecific CatL substrate conversion (~ 30 %) were observed. Only 20 % of the population did not show any sublethal or lethal effects at 2.5 µM DNOC (approx. EC_{80}).

For Rotenone also concentration dependent effects on specific, unspecific and thus total CatH substrate conversion were observed (Figure 6-8 C). But efficiency with maximal 50 % for unspecific CatH substrate conversion and around 20 % for specific CatH activity was lower at the EC_{80} of Rotenone (15 nM) compared to EC_{80} of DNOC (see above). No concentration dependent effects were detectable for CatL or CatC substrate conversion for Rotenone.

Ethanol, in contrast to Rotenone and DNOC, caused dose-dependent induction of CatC (Figure 6-8 B). Interestingly, effects on CatH and CatL substrate conversion were not detectable for Ethanol.

6.3 Discussion

Results of Chapter 4 have shown that toxic stress can impair yolk consumption processes in zebrafish embryos. For teleost embryos and other oviparous animals, the proteases of the cathepsin-type have been proposed as the major yolk degradation enzymes (Carnevali *et al.* 1999, 2001, 2006, Fagotto 1990, Kwon *et al.* 2001, Sire *et al.* 1994, Qiu *et al.* 2005, Yoshizaki and Yonezawa 1994). Hence, this study aimed to investigate the potential of cathepsins to serve as a novel group of biomarkers indicating disturbed yolk consumption and thus development processes in zebrafish embryos subdued to toxic stress. Stegemann (1992) and Mayer *et al.* (1992) have reviewed and proposed various criteria which should be met by candidate biomarkers. Hence, in

relation to these requirements, the data of this study will be discussed concerning technical, biological validation as well as toxicological aspects to characterise the potential of cathepsins as novel biomarkers.

6.3.1 Cathepsin L, C and H Activity During Embryogenesis

For *Danio rerio* embryos, the enzymatic role of cathepsins in yolk processing has not been studied so far. Therefore, activities of different cathepsins were analysed in zebrafish eggs. We described for the first time activities of the three cystein proteases CatL, CatC and CatH in the *Danio rerio* eggs. These findings are supported by results of Tingaud-Sequeira and Cerda (2007) who observed mRNA of CatL and CatC in the yolk syncytical layer of *Danio rerio* embryos and by Farba and Cerda (2004) showing CatH, CatL and CatC mRNA in oocytes of the teleost *F. heteroclitus.* Moreover, for *Danio rerio*, entries for all three proteins could be identified in the UniProKB/swiss-Prot and TrEMBL database. Whereas important functions for CatL and CatH in yolk degradation have been described for several oviparous animals including fish, activity of CatC in eggs was only observed for crustacean species so far (Qiu *et al.* 2005). Hence, our results would support a role of CatC in yolk consumption in fish species, as well. This is further supported by our finding that CatC showed activity rates comparable to CatL which is considered to exert the predominant cathepsin activity in the yolk during embryogenesis of several fish species (Carnevali *et al.* 1999, Fagotto 1995, Sire *et al.* 1994). Compared to CatC and CatL, CatH showed 30 times lower activities pointing to a minor but possibly specific function of CatH in yolk processing of *Danio rerio* embryos. Overall, it can be concluded, like in other teleost species, that cathepsins exert important functions during embryogenesis of zebrafish. Moreover, the observation of activity of more than one cathepsin and their different activities support the hypothesis that the yolk enzymatic machinery is an interaction of various enzymes which are strongly regulated. Hence, the whole set of cathepsins should be considered as novel biomarker group.

Cathepsins belong to a conserved and homologous enzyme family (Fabra and Cerda 2004) and unspecific substrate conversion processes can be well expected. Therefore, specificity of the cathepsin substrate conversion in the applied enzymatic assays will be discussed. Data for specificity were deduced from the total cathepsin substrate conversion processes by the use of specific cathepsin inhibitors. Stable unspecific substrate conversion was detected for CatL and CatH substrates. These could be either due to homologous cathepsins in the samples or also by presence of other

proteases like aminopeptidase B cleaving unspecifically CatH substrate (Barrett 1980). CatC substrate (H-Gly-Arg-AMC) conversion is highly specific for Dipeptidylaminopeptidase I (CatC) (Chan *et al.* 1985) and hence no unspecific reaction was detected and assumed for CatC. All measured specific as well as unspecific enzymatic reactions could contribute to yolk processing (Vtg degradation) and hence should be similarly considered when cathepsin activities are consulted to indicate abnormal yolk degradation processes.

To distinguish between natural variability and contaminant-induced stress, baseline data for the activity of candidate biomarkers are indispensable (Stegemann *et al.* 1992). Therefore, cathepsin activities at different stages of embryonal activity were studied. All measured cathepsin activities were very low in the first 10 hpf which is a confirmation of results of Chapter 3 showing lower enzymatic activities in the early blastula and gastrula stages. A strong increase of CatH, L and C substrate conversion rates occurred in the segmentation period until 26 hpf. This is consistent with dramatic changes in the yolk protein pattern of *Danio rerio* embryos during that period of development (Chapter 3). Reasons for increasing yolk degradation activities might be found in the beginning of embryonal organ and somite formation within this period leading to changed energy and nutrient metabolism in the embryos (Kimmel *et al.* 1995). In the later developmental stages all measured cathepsin activities showed different temporal progressions. To summarise, our findings point to time-specific and regulated enzymatic processes in yolk degradation which were also anticipated by several publications (Carnevali *et al.*1999, 2001, 2006, Fabra and Cerda *et al.* 2004, Kwon *et al.* 2001, Sire *et al.* 1994) Moreover, they support the finding that in contrast to cellular lysosomes which rapidly digest all proteins down to free amino acids, yolk lysosomes show time-dependent activities and do not degrade their contents until later developmental stages (Carnevali *et al.* 1999, Fabra and Cerda 2004, Fagotto 1995). Hence, it can be concluded that synchronisation of cathepsin activities to embryonal stages is of concern for correct baseline data.

Cathepsins have long been known to exert the major enzymatic activities in cellular lysosomes for the normal cellular protein turn over (McGrath 1999). As we have not investigated the spatial distribution of the cathepsins activities in the developing embryos we can not exclude additional enzymatic activities from the classical intracellular lysosomal functions in the embryos. But appearance of cathepsin activities shortly after fertilisation of the eggs, when only few embryonal cells are present, and a

decline of the cathepsin activities in 5 dpf old larvae hint to activities of the measured cathepsins in the yolk. In addition, as was mentioned above, mRNA of CatL and CatC was detectable in the yolk syncytical layer of *Danio rerio* embryos (Farba and Cerda 2004). From this can be concluded that the measured cathepsin activities are associated with the yolk.

Easy measurement methods are another demand in terms of biomarker development noted by Stegeman *et al.* (1992). Hence, all applied cathepsin assays were based on 7-Amino-4-methylcoumarin as fluorescent reaction product, performed under the same enzymatic conditions and optimised for 96-well microplates. Therefore, multiple cathepsin assays are performable on the same 96-well plates and could thus have potential for high-through-put applications.

To summarise, several cathepsin activities, associated with yolk degradation, were found in zebrafish eggs showing continuous but time- and enzyme- dependent rates during the embryonal development. These findings support the hypothesis that a whole set of regulated cathepsins is involved in yolk processing and hence they could be considered and analysed as a whole group of potential biomarkers. However, the understanding of the specific roles of the different enzymes in the yolk protein cleavage processes remains to be clarified.

6.3.2 Toxicant Related Changes in Cathepsins Activities

Toxicity related changes in cathepsins activities were studied using the four different chemicals Leupeptin, Rotenone, DNOC and Ethanol. Leupeptin is a known CatL inhibitor (Aoyagi *et al.* 1969) and was hence chosen as a reference toxicant to validate the applied system and to test for effects resulting from specific cathepsin inhibition. For Rotenone and DNOC specific *Modes of Action* within the oxidative phosphorylation and as decoupler, respectively, are described (Schuler and Casida 2001, Pelfrene 2000). They can be directly related to effects caused by disturbances in energy metabolism and were chosen because direct links between compromised energy metabolism and yolk consumption including cathepsin activities were assumed. Ethanol generally affects embryogenesis causing numerous rather unspecific effects (Loockwood *et al.* 2004). For the three substances only indirect interaction with cathepsin activities are expected because *in vivo* inhibition of cathepsins by Ethanol is improbable (Mantle *et*

al. 1999) and no literature data was available hinting to *in vivo* cathepsin inhibition by DNOC and Rotenone.

In the following, toxicity related aspects of cathepsins as novel biomarkers will be discussed. For all four substances substantial effects on cathepsin activities were detectable. Thus we can conclude that abnormal cathepsin activities in zebrafish eggs indicate toxic exposure and that they could be biomarkers integrating the indication of exposure to diverse substances. Furthermore, for all substances, effects on cathepsin activities increased with rising concentrations demonstrating clear concentration-dependence of the novel biomarker family. To characterise the sensitivity, effects on cathepsin activities were compared to established sublethal endpoints for zebrafish embryos. Sensitive biomarkers indicate effects at earlier time points or lower exposure levels compared to traditional toxic endpoints (Chambers *et al.* 1994). As only apparently intact organisms were included in the enzymatic analyses, we conclude that cathepsins indicate toxic stress early at the molecular level. In addition, reactions in cathepsin activities were detectable at exposure levels not leading to any morphologically discernible effects for all substances. Therefore, cathepsin activities might be a more sensitive toxic endpoint for *Danio rerio* embryos compared to the classical endpoints described for DarT (Nagel 2002). All tested substances led to similar sublethal effects in lower concentration, including oedema, impaired blood-circulation or retardation of development. Changes in cathepsin activities might precede these effects and hence, next to being a biomarker indicating toxic exposure, they may be also interpreted as biomarkers indicating early sublethal effects. Except for Leupeptin, increasing effects to cathepsin activities were followed by a rising frequency of the occurrence of sublethal effects. Hence, changed levels of cathepsin activities could be correlated with the health status and fitness of the organisms. To summarise, cathepsin activity levels changed dose-dependently for several toxicants and are interpreted as biomarkers possibly indicating toxic exposure and early sublethal effects. As biomarkers they demonstrate many properties that are requested for candidate biomarkers (Stegeman *et al.* 1992).

As expected, the specific cathepsin inhibitor Leupeptin led to the highest efficiency and potency compared to the other substances. Inhibition of Leupeptin was already detectable at 100 fold lower exposure levels compared to occurrence of microscopically visible effects. However, embryos could cope with high CatL inhibition at 0.1 mM Leupeptin (only 10 % of embryos with microscopically visible effect at 95 % CatL inhibition) even if clear effects on the yolk protein pattern at this concentration

were detectable. Hence, for cathepsin inhibitors like Leupeptin, cathepsin activity would be a suitable marker for sensitive detection of toxic stress. Furthermore, this might also be of ecotoxicological interest for risk assessment of pharmaceuticals in future because cathepsins are related to many human diseases and are targeted in pharmaceutical research (Baricos *et al.* 1991, Cunnane *et al.* 1999, Maehr *et al.* 2005, Saegusa *et al.* 2002, Que and Reed 2000).

Even as all tested substances affected cathepsin activities, differences in the patterns of cathepsin interference were discernible with respect to the type and the number of types of affected cathepsins, the induction -or inhibition processes, and also regarding differences in potency and efficiency. This gives room for interpreting different exposure scenarios against different toxicants by analysing cathepsin activities and may hint to substance specificity of the novel biomarker group.

Some findings in the effect pattern of cathepsins affection for the four substances were quite astonishing. For some fish species, CatL has been described as exerting the major enzymatic activity in the yolk during embryogenesis (Carnevali *et al.* 1999, Fagotto 1995, Sire *et al.* 1994). In this study however, except for Leupeptin, no effects on CatL activity were observed. In fact, Ethanol, DNOC and Rotenone affected CatH or CatC substrate conversion processes most. This could point to an important role of these enzymes in yolk processing and emphasises the importance of investigation of different enzymes of the whole cathepsins family.

Leupeptin, DNOC and Rotenone caused decreased levels of cathepsins activities. This can well be expected because it indicates an impairment in energy metabolism, yolk processing and hence retardation in embryonal development. These assumptions are confirmed by protein analysis of Leupeptin treated embryos which showed retardations in yolk processing. For Ethanol, the findings are however contradicting. Even if proteome studies with Ethanol have revealed retarded Vtg processing (Chapter 3) in *Danio rerio* embryos, Ethanol was the only substance causing increased proteolysis which actually would hint to faster yolk degradation and nutrient releasing processes. One explanation could be that the critical cathepsin type was not tested for Ethanol; but as the biological functions of cathepsins are still unclear, interpretation of the results are so far very speculative. In general, increased cathepsin levels might be interpreted as compensatory for other yolk proteolysis impairment processes and/or may reflect the need of definite nutrients necessary in stress response.

Surprisingly, some effects on proteolysis were similar for DNOC and Rotenone exposure. Both substances reduced CatH substrate conversion, which might be seen in connection to their MOAs in the oxidative phosphorylation.

Biochemical interpretation of the affected "cathepsins patterns", observed for the different substances, can not be taken any further, because the specific roles of cathepsins are largely unknown, but they show, that cathepsins hold the potential to indicate substance and *Mode of Action* specific exposure. Future research should focus to systematise the types of exposure and effects cathepsins could reliably indicate. Overall, it can be assumed that the observed complexity in toxic effects to cathepsins may be a mirror of complex yolk degradation processes.

Toxic stress is always related to energy metabolism and a wide group of substances might influence nutrient release from yolk and hence cathepsin activities in developing oviparous animals. Moreover, it is accepted that impairments in the strictly regulated yolk degradation processes may lead to disorders in embryonal development (Fagotto 1995, Kanaya *et al.* 2000). But, cathepsins as yolk degradation enzymes have not been seen as biomarkers so far and only few studies are available having investigated the relation of chemical exposure and cathepsin activity (Kaivarainen *et al.* 1989, Maradonna and Carnevali 2007, Yang *et al.* 2006, Wood *et al.* 2003). Hence, this study for the first time shows the high potential of a whole enzymatic family to serve as novel biomarkers.

In conclusion, for microscopically intact *Danio rerio* embryos, concentration dependent effects of four substances on yolk enzymatic activity, responsible for regulated nutrient release, could be shown. Therefore, we propose to regard the yolk enzymes of the cathepsin-type as a novel biomarker. As we observed substance- and *Mode of Action*-specific effects and could also relate the altered cathepsin activities to sublethal effects in the embryos, cathepsins hold the potential to be biomarkers signalling different exposure and effect scenarios. Moreover, yolk enzymes could be a new generation of biomarkers for monitoring normal embryonal development and toxic stress in zebrafish embryos and might be applied as additional toxicological endpoints in the zebrafish embryo assay. In addition, they might be of interest to all oviparous vertebrates. Future research should include the investigation of different cathepsin types, systematise sensitivity against toxicants and study transferability to other oviparous organisms.

CHAPTER 7

Summary and Conlusions

Sensitive effect determination, the understanding of molecular toxicity mechanisms and the discovery of novel biochemical biomarkers are some of the major challenges in ecotoxicology in dealing with chemicals in the environment. These can be addressed by effect analysis at the molecular level in well known ecotoxicological model organisms. Functional genomics techniques, allowing the unbiased and simultaneous characterisation of large groups of biomolecules, were recently introduced in the field of ecotoxicology for molecular effect determination. Among several 'omics' tools, proteomic approaches are used to study the whole proteome of organisms and may provide novel insights into the functional molecular state of a biological system. The use of early life stages of the model organism zebrafish in ecotoxicoproteomics approaches is very promising due to many properties including a nearly sequenced genome, a fast embryonal development, no external feeding and the high number of available organisms. Moreover, they are proposed for usage as replacement of animal experiments. Therefore, the present thesis aimed to make early life stages of the zebrafish accessible to ecotoxicoproteomics applications and to investigate the potential of proteomics approaches with zebrafish embryos for unravelling of molecular mechanisms of toxicity, for discovery of novel sensitive biomarkers indicating exposure or effects and for enabling early effect recognition at low toxicant concentrations. These challenges were addressed in four consecutive steps.

The first objective of the work was to make zebrafish embryos accessible for a 2-DE based ecotoxicoproteomics approach (**Chapter 3**). This included the establishment of appropriate 2-DE conditions for zebrafish embryo protein samples, the characterisation of *Danio rerio* protein identification success with MS based methods after 2-DE separation, as well as the creation of baseline proteomic data at different developmental stages by characterising the changes in the proteome pattern during the embryonal development of zebrafish. Well separated embryonal protein patterns could be achieved for all investigated developmental stages and subsequent MS based identification of the proteins was possible. It was found that, until four to five days after egg fertilisation, the proteome of whole zebrafish embryos is dominated by high abundant yolk proteins, vitellogenin derivatives. Vitellogenins are conserved, very large and highly posttranslationally modified proteins that are produced by the female fish adults and are inoculated into the early oocytes. They ensure the nutrient supply of the embryos during the early development by being proteolytically cleaved into smaller fragments

(vitellogenin derivatives) and by releasing bound nutrients such as amino acids, lipids, carbohydrates, sugars or metal ions. The 2-DE analysis of embryonal protein patterns at different developmental stages in this work revealed specific yolk protein patterns for each developmental stage. This indicates that proteolytic cleavage of yolk proteins is strongly regulated and crucial for normal embryonic development. Hence, it was concluded that altered yolk protein composition might mirror impaired embryogenesis. Westernblot analysis with an anti-vitellogenin-antibody and MS identification results could show that the ratio of cellular proteins to yolk proteins increases during development. In the proteome of five day old larvae, barely any yolk proteins could be detected. Hence, it was concluded that the usage of five day old zebrafish larvae, in contrast to the embryos, could enable toxicoproteomics applications focussing more on cellular proteins.

In conclusion, results of Chapter 3 show that a 2-DE based proteomics approach with developing zebrafish is possible at different developmental stages whereby information extracted from ecotoxicoproteomics applications depend on the embryo or larvae age.

The potential for exotoxicoproteomics applications with zebrafish embryonal or larval stages were further investigated (**Chapter 4 and 5**). To study yolk protein processing impairments, on the one hand, toxic responses in the proteome of 2 dpf old embryos were characterised (Chapter 4). On the other hand, to focus more on cellular proteins, ecotoxicoproteomics approaches with five day old eleutheroembryos (termed as larvae) were carried out (Chapter 5).

For the toxicoproteomics analysis of impairments in yolk protein processing (**Chapter** 4), 2 dpf old embryos were chosen due to two reasons: On the one hand, above results have shown that 2-DE analysis of protein samples from 2 dpf embryos resulted in well separated pattern of a high number of vitellogenin derivatives. On the other hand, due to the *Danio rerio* embryo assay (DarT), microscopically visible sublethal and lethal toxic endpoints are well defined for this developmental stage and would allow the linking of effects on the molecular level (proteome level) to effects at higher levels of biological organisation (physiological or morphological level). Ethanol affects zebrafish embryogenesis causing various effects such as impairment of the cardio-vascular-system and was chosen as a reference toxicant for a toxicoproteomics approach in 2 dpf old embryos. Indeed, Ethanol exposure was detectable at the proteome level as it provoked significantly changed yolk protein patterns probably

caused by impairments in the vitellogenin proteolytic processing. It was suspected that toxicant exposure costs energy and leads to an altered energy metabolism which can be directly seen in nutrient usage and hence, in yolk protein processing. Altered vitellogenin fragmentation patterns were already detectable in treated organisms that did not show any morphological effects. Therefore, analysis of vitellogenin cleavage patterns might be useful as a sensitive diagnostic tool to indicate toxicant stress response in zebrafish embryos at the molecular level. This could be used to predict effects at higher organisation levels, at increased toxicant concentrations, and to monitor the overall fitness of intact organisms.

The potential of exploring molecular mechanisms and of discovery of candidate biomarkers focussing on more cellular proteins by a proteomics approach in 5 dpf old larvae, was investigated in **Chapter 5**.

Five dpf old larvae begin with external feeding and results of Chapter 3 have shown that most of the yolk proteins are degraded down to free amino acids and do not hamper the analysis of cellular proteins. In younger embryos, analysis of cellular proteins was shown to be only possible by application of manual deyolking techniques. In an ecotoxicoproteomics approach, however, these techniques may interfere with the detection of stress-induced responses by the test compounds. Hence, in this work, the usage of larvae was described as a possibility to overcome deyolking techniques and to enable effect analysis at the cellular protein level. Ecotoxicoproteomics approaches in larvae were performed using three reference substances with known *Modes of Action*, two toxicants interfering with the energy metabolism, Rotenone and DNOC, and one anti-inflammatory pharmaceutical, Diclofenac. All toxicants induced significant changes at the proteome level in organisms not showing effects at higher organisation levels (by microscopical inspection). The patterns of significantly changed proteins were substance and concentration dependent. Additionally, some proteins unspecifically changed in expression after treatment with all three substances. Moreover, for Rotenone and DNOC, substances with *Modes of Action* in oxidative phosphorylation, the induced changes in the protein pattern were more similar compared to Diclofenac. Hence, it was concluded that a toxicoproteomics approach in zebrafish larvae enables the detection of substance specific effects and more general stress responses in the cellular protein pattern and allows the identification of candidate protein biomarkers. Of course, subsequent identification of the proteins of interest is

necessary to relate these proteins to biochemical pathways to obtained insights in the molecular mechanisms of toxicity and to establish them as novel biomarkers.

The last part of the thesis (**Chapter 6**) addresses the establishment of novel biomarkers derived from the applied ecotoxicoproteomics approaches described in the chapters before. The identification of candidate biomarkers was based on the finding that toxicant exposure induced changes in yolk protein patterns by impairing the vitellogenin proteolytic processing (Chapter 4). For fish, the lysosomal endoproteases cathepsins, are described to be involved in the vitellogenin processing. Hence, the aim of the last part of the thesis was to investigate if cathepsins activities might be suitable novel biomarkers of toxic exposure. Therefore, cathepsin activities in developing zebrafish embryos were characterised under normal and toxic exposure conditions. *In vivo* activities of three Cathepsins L, H and C were analysed at different developmental stages of zebrafish embryos. In addition, their responses to exposure of fish embryos against four reference toxicants Leupeptin, Ethanol, Rotenone and DNOC, was studied. Continuous activities of Cathepsins L, H and C could be observed in the embryos showing developmental stage dependent activity rates. For all tested toxicants, clear concentration dependent and toxicant specific effects on the cathepsin activities in microscopically intact zebrafish embryos were detectable. Hence, it was concluded that cathepsins may become a new generation of biomarkers for monitoring normal embryonal development and toxic stress in zebrafish embryos and might be also applied as novel sensitive toxicological endpoints in the zebrafish embryo assay (DarT). In addition, they could be transferable to many oviparous vertebrates because cathepsins are assumed to be general yolk processing enzymes in all oviparous vertebrates.

In conclusion, the results of this thesis enabled the establishment of toxicoproteomics approaches in early life stages of the zebrafish. For the first time, yolk utilisation processes were associated with toxicant exposure. Moreover, treatment of zebrafish larvae with low concentrations of different toxic compounds was detectable and distinguishable at the proteome level in intact organisms (not showing microscopically visible effects). Hereby, proteins specificly or unspecificly responding to toxic stress were found. Furthermore, with the enzyme family of cathepsins, a novel candidate biomarker group for the indication of toxic exposure was identified.

Therefore, the results of this thesis demonstrate and confirm the high potential of ecotoxicoproteomics with zebrafish for sensitive effect detection at low toxicant concentrations, for obtaining insights in molecular processes underlying toxicity, and for discovery of novel biochemical biomarkers.

Of course, some research is still necessary to elaborate the powerful tool of toxicoproteomics with early life stages of zebrafish for routine usage to identify new toxicity targets, discover novel biochemical biomarkers or for hazard characterisation. This includes the investigation of the robustness of the toxic stress response concerning different methodological or statistical analyses tools for the proteomics data, an optimised system to enable fast and high-throughput identification of proteins of interest, the testing with various chemicals with known *Mode of Actions* and also the establishment of strategies enabling the characterisation of low abundant protein classes.

As results of this thesis contribute to a deeper understanding of developmental and toxicity processes in a well described vertebrate organism, this work might, next to ecotoxicology, also be taken as a support in other scientific fields like developmental biology or human toxicology. In addition, the proteomics research on embryonal stages of the zebrafish could help to advance the replacement of experiments with adult fish.

Zusammenfassung

Frühe Effektdiagnose bei geringen Schadstoffkonzentrationen, das Verstehen molekularer Toxizitätsmechanismen und die Entdeckung neuer biochemischer Biomarker sind einige der Hauptaufgaben in der ökotoxikologischen Forschung, um die Toxizität von Chemikalien in der Umwelt besser verstehen und bewerten zu können. Einen Weg hierfür bietet Effektanalyse auf molekularer Ebene. Für die Effektdetermination auf molekularer Ebene wurden erst kürzlich so genannte ‚Omics'-Techniken in das Gebiet der Ökotoxikologie eingeführt, da sie eine unvoreingenommene und gleichzeitige Charakterisierung großer Biomolekülgruppen erlauben. Unter den verschiedenen ‚Omics' Ansätzen, ermöglicht Proteomics die Analyse des gesamten Proteoms eines Organismus und kann somit Einblicke in den eher funktionellen Status eines biologischen Systems liefern. Der ökotoxikologische Modellorganismus Zebrafischembryo ist aufgrund seiner Größe, seiner schnellen Embryonalentwicklung und seines fast vollständig sequenzierten Genoms viel versprechend für Proteomics-Anwendungen. Das Ziel der vorliegenden Doktorarbeit war daher, Toxizität basierte Proteomics-Analysen mit dem Zebrafischembryo zu etablieren, um Einblicke in Wirkmechanismen von toxischen Substanzen zu erlangen, neuartige Biomarker zur Indikation von toxischem Stress zu finden und frühe Effektdiagnose zu ermöglichen. Verschiedene Aspekte dieser Aufgabe wurden in vier aufeinander aufbauenden Kapiteln behandelt.

Im ersten Teil der Arbeit sollten Zebrafischembryonen zu einem Proteomics Ansatz, basierend auf zwei-dimensionaler Gelelekrophorese (2-DE), zugänglich gemacht werden (**Kapitel 3**). Dazu gehörten zu einem die Etablierung von geeigneten 2-DE Bedingungen für Proteinproben des Zebrafisch Embryos und zum anderen die Untersuchung des embryonalen Proteoms im Verlauf der Embryonalentwicklung (Blastula Stadien bis hin zu frühen Larven Stadien). Gut aufgelöste Proteinmuster konnten für alle analysierten Entwicklungsstufen erreicht werden. Eine anschliessende MS basierte Identifikation der *Danio rerio* Proteine war ebenso möglich. Das embryonale Proteom ist in den ersten 3 bis 4 Tagen nach Eibefruchtung von hoch abundanten Dottersackproteinen, den so genannten Vitellogeninderivaten, dominiert. Vitellogenine sind große, hoch konservierte und stark posttranslational modifizierte Proteine, die zur Nährstoffversorgung des Embryos dienen. Sie werden proteolytisch nach und nach in kleinere Fragmente (Vitellogeninderivate) gespalten und setzen dabei

Nährstoffe wie Aminosäuren, Lipide, Kohlenhydrate oder Metallionen frei, welche dann dem sich entwickelnden Embryo zur Verfügung stehen. Für jede analysierte Entwicklungsstufe konnten spezifische Muster an Vitellogeninspaltprodukten detektiert werden. Das indiziert eine streng regulierte proteolytische Prozessierung der Dotterproteine, die für eine normale Embryonalentwicklung entscheidend zu sein scheint. Es wurde geschlussfolgert, dass Veränderungen in der Dottersack-Proteinzusammensetzung auf beeinträchtigte Embryogenese hindeuten können. Im Laufe der embryonalen Entwicklung nehmen Dottersack-Proteine ab. Das konnte mit Hilfe von Westernblot-Analysen mit einem Anti-Vitellogenin-Antikörper und MS Identifizierungsergebnisse gezeigt werden. In dem Proteom von 5 Tage alten Larven konnten kaum noch Vitellogeninderivate detektiert werden. Somit bietet die Verwendung von 5 Tage alten Larven, im Gegensatz zu Embryonen, die Möglichkeit eines Proteomics-Ansatzes mit Fokus auf die Charakterisierung von zellulären Proteinen.

Das Potential von Proteomics-Ansätzen mit Embryonen oder Larven zur Detektion von Toxizität bedingten Veränderungen sollte im weiteren Verlauf der Arbeit charakterisiert werden (Kapitel 4 und Kapitel 5).

Um toxizitätsbasierte Beeinträchtigungen in der Dotterprotein-Prozessierung zu analysieren (**Kapitel 4**), wurde das Entwicklungsstadium 2 Tage nach Eibefruchtung ausgewählt, da eine große Zahl von Vitellogeninderivaten aufgelöst werden konnte. Ethanol ist bekanntermaßen embryotoxisch und verursacht vielfältige Effekte in Zebrafischembryonen. Daher wurde Ethanol als Referenzsubstanz für einen Toxicoproteomics-Ansatz in 2 Tage alten Embryonen ausgewählt. Ethanol-Exponierung verursachte signifikante Veränderungen im Dotterproteinmuster der Embryonen, welche vermutlich auf beeinträchtigte Dotterprotein-Proteolyse zurückzuführen sind. Es wurde angenommen, dass Schadstoffexponierung vielfältige Effekte auf physiologischer und biochemischer Ebene verursacht, die zu einem veränderten Energiebedarf führen und sich direkt auf den Energiemetabolismus, den Nährstoffverbrauch und so die Dotterprotein-Prozessierung auswirken. Veränderte Vitellogenin-Fragmentmuster konnten schon in Ethanol exponierten Embryonen detektiert werden, die keine mikroskopisch sichtbaren Effekte aufwiesen. Daher könnte die Analyse von Dotterproteinmustern die sensitive Diagnose von Schadstoffstress in Zebrafischembryonen ermöglichen, was zur Vorhersage von Effekten bei höheren

Schadstoffkonzentrationen und auch zur generellen Charakterisierung der ‚Fitness' eines Organismus dienen könnte.

In **Kapitel 5** wurde das Potential eines Toxicoproteomics-Ansatzes in Zebrafischlarven untersucht. Am fünften Tag nach der Eibefruchtung beginnt die externe Ernährung der Zebrafischlarven. Ergebnisse aus Kapitel 3 haben gezeigt, dass zu diesem Stadium fast alle hoch abundanten Vitellogeninderivate zu freien Aminosäuren degradiert sind. Daher verhindern diese in 5 Tage alten Larven nicht mehr die Analyse von zellulären Proteinen. Drei Referenzsubstanzen mit bekanntem Wirkort, Rotenon und DNOC, mit Wirkung in der oxidativen Phosphorylierung und Diclofenac, als Cyclooxygenase Inhibitor, wurden für die Toxicoproteomics-Experimente mit Larven ausgewählt. Alle drei Schadstoffe verursachten signifikante Veränderungen im Proteom von äußerlich intakten Larven. Die Muster der differentiell exprimierten Proteine waren dabei sowohl substanz- als auch konzentrations-abhängig. Allerdings konnten auch Proteine detektiert werden, die bei allen drei Schadstoffen differentiell reguliert wurden. Die Veränderungen im larvalen Proteom, welche durch Rotenon und DNOC, zwei Stoffe mit Wirkort im gleichen biochemischen Stoffwechselweg, verursacht wurden, waren ähnlicher verglichen mit Diclofenac. Daher wurde geschlussfolgert, dass ein Toxicoproteomics Ansatz mit Larven Exposition verschiedener Substanzengruppen auf Proteinebene indizieren kann. Nach erfolgreicher Identifikation der differentiell exprimierten Proteine, könnten Einblicke in Toxizitätsmechanismen und auch die Entdeckung potentieller Biomarker möglich sein.

Im letzten Teil der Arbeit (**Kapitel 6**) sollte versucht werden, einen potentiellen Biomarker aus den durchgeführten Toxicoproteomics Ansätzen zu entwickeln. Die Identifizierung eines neuartigen Biomarkers basierte hierbei auf den Ergebnissen aus Kapitel 4, welche zeigten, dass Schadstoffstress zu beeinträchtigter Proteolyse der Dotterproteine führen kann. Cathepsine, lysosomale Endoproteasen, sind in die proteolytische Prozessierung der Vitellogenine in Fischembryonen involviert. Demnach sollte überprüft werden, ob sich Cathepsine als Biomarker zur Indikation von Schadstoffexposition eignen. Hierfür wurden Cathepsin Aktivitäten in Embryonen unter Kontrollbedingungen und Schadstoffexposition analysiert. *In vivo* Aktivitäten für Cathepsin L, H und C wurden in verschiedenen Entwicklungsstadien charakterisiert und deren Reaktion auf Exponierung mit vier Referenzsubstanzen (DNOC, Rotenon,

Leupeptin und Ethanol) untersucht. Entwicklungsstadium abhängige, aber kontinuierliche Aktivitäten konnten für Cathepsin L, H und C in sich entwickelnden Zebrafischen bis zum 5. Tag nach Eibefruchtung detektiert werden. Zusätzlich wurden für alle getesteten Substanzen klare konzentrationsabhängige und schadstoffspezifische Effekte in den Cathepsin Aktivitäten in mikroskopisch intakten Embryonen gefunden. Daraus wurde geschlossen, dass Cathepsine eine neue Biomarkerfamilie darstellen könnten, um embryonale Entwicklungsprozesse zu kontrollieren und Schadstoffstress in Zebrafisch Embryonen zu indizieren. Sie könnten daher auch als neue sensitive toxische Endpunkte im Zebrafisch Embryo Test Anwendung finden. Im Allgemeinen könnte der Biomarker Cathepsin auf alle oviparösen Organismen übertragbar sein, da Cathepsine als generelle Dotterprotein Proteolyse Enzyme in allen Eier-produzierenden Organismen beschrieben werden.

Die Ergebnisse dieser Doktorarbeit haben die Etablierung von Proteomics in frühen Lebensstadien von Zebrafischen ermöglicht. Zum ersten Mal konnten Dotterproteine mit Schadstoffexposition in Verbindung gebracht werden. Des Weiteren konnten Exponierung mit verschiedenen Schadstoffen auf Proteinebene von Larven detektiert und Proteine, die spezifisch oder generell auf toxischen Stress reagiert haben, charakterisiert werden. Effekte waren hierbei in mikroskopisch intakten Organismen detektierbar. Mit der Enzymfamilie Cathepsin, konnten weiterhin neue potentielle Biomarker zur Indikation von Schadstoffstress in Fischembryonen identifiziert werden. Daher verdeutlichen und zeigen Ergebnisse dieser Arbeit das hohe Potential von Ecotoxicoproteomics in Zebrafischen für sensitive Effektermittlung, für die Aufklärung von Wirkmechanismen und das Finden neuartiger Biomarker.

Allerdings ist noch mehr Forschungsbedarf nötig, um Toxicoproteomics mit Zebrafischembryonen und -larven für Routineanwendungen zur Identifikation neuer Biomarker, zur Aufklärung von Wirkmechanismen und zur frühen Effektdiagnose, zu vervollkommnen. Dies schliesst zum Beispiel den Vergleich verschiedener statistischer Analysemethoden für Proteomics-Daten, die Optimierung der Protein-Identifikation, das Testen von verschiedenen Schadstoffen mit bekanntem Wirkort und auch Möglichkeiten, um verschiedene Proteinklassen zu analysieren, ein.

Da die Ergebnisse dieser Arbeit zu einem tieferen Verständnis von Entwicklungsprozessen und Toxizitätsmechanismen in einem gut beschriebenem Modellorganismus beitragen, liefert diese Arbeit auch Beiträge zu wissenschaftlichen

Disziplinen außerhalb der Ökotoxikologie wie Entwicklungsbiologie oder und Humantoxikologie. Weiterhin könnten Proteomics Untersuchungen an Embryonalstadien des Zebrafischs die Entwicklung von Ersatzmethoden für Experimente mit adulten Fischen unterstützen.

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Appendix

Contents

I Results of Toxicoproteomics Studies (III)

Table A1 Toxicoproteomics experiment with Ethanol treated zebrafish embryos: Spot IDs. mean values and statistical analysis of all significantly over-expressed proteins after 2 % ethanol exposure and also their values after 0.5 % ethanol exposure: background shading indicates spots with a significant ($p<0.05$) at least two-fold difference in abundance after also 0.5 % ethanol treatment.

spot ID	*mean value controls*	*mean value 0.5 % v/v ethanol*	*mean value 2 % v/v ethanol*	*ratio 0.5 % ethanol /control*	*(significance, t-test result)*	*ratio 2 % ethanol /control*	*(significance, t-test result)*
oe1	0.0011	0.0210	0.0860	19.81	(0.01)	81.37	(<0.01)
oe2	0.0032	0.0355	0.2316	11.23	(0.02)	73.20	(<0.01)
oe3	0.0076	0.1602	0.5078	21.21	(0.05)	67.26	(<0.01)
oe4	0.0008	0.0146	0.0527	18.59	(0.04)	67.05	(<0.01)
oe5	0.0066	0.0870	0.3907	13.16	(0.01)	59.12	(<0.01)
oe6	0.0016	0.0161	0.0782	10.13	(0.02)	49.27	(0.03)
oe7	0.0056	0.0380	0.2344	6.75	(0.01)	41.68	(<0.01)
oe8	0.0025	0.0181	0.0929	7.21	(0.03)	37.02	(<0.01)
oe9	0.0051	0.0228	0.1249	4.45	(0.05)	24.42	(0.01)
oe10	0.0074	0.0553	0.1728	7.51	(0.01)	23.44	(<0.01)
oe11	0.0044	0.0205	0.1017	4.68	(0.02)	23.24	(<0.01)
oe12	0.0054	0.0687	0.1170	12.61	(0.06)	21.50	(<0.01)
oe13	0.0027	0.0019	0.0563	-1.38	(0.82)	21.16	(<0.01)
oe14	0.0529	0.4115	0.7465	7.78	(0.03)	14.11	(<0.01)
oe15	0.0047	0.0186	0.0663	3.92	(0.10)	13.99	(<0.01)
oe16	0.0130	0.0498	0.1741	3.82	(0.07)	13.36	(<0.01)
oe17	0.0188	0.0735	0.2050	3.91	(0.07)	10.92	(<0.01)
oe18	0.0111	0.0233	0.1164	2.11	(0.40)	10.52	(<0.01)
oe19	0.0435	0.2682	0.4322	6.16	(0.04)	9.93	(<0.01)
oe21	0.0209	0.0423	0.1875	2.02	(0.22)	8.95	(<0.01)
oe22	0.0281	0.0754	0.2141	2.68	(0.08)	7.62	(<0.01)
oe25	0.0502	0.0853	0.2957	1.70	(0.12)	5.89	(0.01)
oe26	0.0166	0.0121	0.0971	-1.37	(0.62)	5.85	(0.01)
oe27	0.1226	0.3529	0.6771	2.88	(0.10)	5.52	(<0.01)
oe28	0.0368	0.0629	0.2011	1.71	(0.35)	5.46	(0.01)
oe29	0.0464	0.0959	0.2509	2.07	(0.19)	5.41	(<0.01)
oe30	0.0290	0.0526	0.1560	1.81	(0.37)	5.38	(<0.01)
oe31	0.0324	0.0501	0.1661	1.55	(0.49)	5.13	(<0.01)
oe32	0.0872	0.2390	0.4432	2.74	(0.09)	5.08	(<0.01)
oe33	0.0377	0.1621	0.1881	4.30	(0.04)	4.99	(<0.01)
oe34	0.0966	0.1879	0.4538	1.95	(0.20)	4.70	(<0.01)
oe35	0.0583	0.1052	0.2667	1.81	(0.26)	4.58	(<0.01)
oe36	0.0721	0.1077	0.3112	1.50	(0.49)	4.32	(<0.01)
oe37	0.0717	0.1886	0.2906	2.63	(0.13)	4.05	(<0.01)
oe38	0.0820	0.1275	0.3196	1.56	(0.25)	3.90	(<0.01)
oe39	0.0754	0.1029	0.2761	1.37	(0.51)	3.66	(<0.01)
oe40	0.0381	0.0610	0.1391	1.60	(0.34)	3.65	(<0.01)
oe41	0.1773	0.2849	0.6419	1.61	(0.13)	3.62	(<0.01)
oe43	0.0383	0.0674	0.1341	1.76	(0.28)	3.50	(<0.01)
oe45	0.0437	0.0627	0.1490	1.44	(0.53)	3.41	(0.01)
oe47	0.0370	0.0377	0.1182	1.02	(0.98)	3.20	(0.02)
oe48	0.0733	0.0938	0.2341	1.28	(0.57)	3.20	(<0.01)
oe49	0.0951	0.1565	0.3035	1.65	(0.09)	3.19	(0.02)
oe50	0.0780	0.1307	0.2416	1.68	(0.24)	3.10	(<0.01)
oe51	0.1705	0.2390	0.5247	1.40	(0.29)	3.08	(<0.01)
oe52	0.1313	0.1533	0.4019	1.17	(0.71)	3.06	(<0.01)
oe53	0.0679	0.0463	0.2009	-1.46	(0.32)	2.96	(0.01)
oe54	0.1065	0.1508	0.3108	1.42	(0.30)	2.92	(0.01)

oe57	0.1367	0.2027	0.3866	1.48	(0.18)	2.83	(<0.01)
oe58	0.1178	0.0427	0.3286	-2.76	(0.03)	2.79	(<0.01)
oe59	0.0636	0.0950	0.1771	1.49	(0.44)	2.78	(0.01)
oe60	0.0778	0.1185	0.2108	1.52	(0.25)	2.71	(<0.01)
oe61	0.3550	0.2650	0.9194	-1.34	(0.29)	2.59	(<0.01)
oe62	0.0707	0.0901	0.1830	1.27	(0.53)	2.59	(0.02)
oe63	0.2856	0.3066	0.7375	1.07	(0.82)	2.58	(<0.01)
oe64	0.0430	0.0898	0.1617	2.09	(0.22)	3.76	(0.01)
oe65	0.2163	0.3343	0.5389	1.55	(0.38)	2.49	(0.01)
oe66	0.1310	0.1299	0.3264	-1.01	(0.98)	2.49	(0.02)
oe67	0.1505	0.1982	0.3606	1.32	(0.41)	2.40	(0.01)
oe68	0.1000	0.1763	0.2357	1.76	(0.18)	2.36	(0.02)
oe69	0.2409	0.3151	0.5547	1.31	(0.52)	2.30	(<0.01)
oe71	0.1518	0.2334	0.3489	1.54	(0.11)	2.30	(<0.01)
oe73	0.1644	0.2101	0.3726	1.28	(0.46)	2.27	(<0.01)
oe74	0.0553	0.0480	0.1246	-1.15	(0.68)	2.25	(0.01)
oe75	0.0512	0.1038	0.1128	2.03	(0.06)	2.20	(<0.01)
oe76	0.0538	0.0722	0.1177	1.34	(0.40)	2.19	(0.04)
oe78	0.1512	0.1595	0.3177	1.06	(0.69)	2.10	(<0.01)
oe79	0.4577	0.2883	0.9604	-1.59	(0.11)	2.10	(<0.01)
oe80	0.3021	0.3507	0.5849	1.16	(0.59)	1.94	(0.02)
oe81	0.1916	0.1596	0.3846	-1.20	(0.49)	2.01	(0.01)
oe86	0.1432	0.3459	0.3621	2.42	(0.03)	2.53	(0.02)
oe87	0.0172	0.0132	0.0732	-1.30	(0.85)	4.26	(0.02)
oe88	0.0324	0.0650	0.1249	2.01	(0.30)	3.85	(0.01)
oe89	0.0069	0.0127	0.0432	1.84	(0.56)	6.29	(0.01)
oe90	0.0349	0.0568	0.0986	1.63	(0.19)	2.83	(0.02)
oe91	0.1330	0.2491	0.4731	1.87	(0.24)	3.56	(0.02)
oe92	0.0032	0.0023	0.0142	-1.36	(0.79)	4.44	(0.04)
oe93	0.0936	0.1096	0.2208	1.17	(0.70)	2.36	(0.02)
oe94	0.0397	0.0685	0.1257	1.73	(0.27)	3.17	(0.01)
oe95	0.0683	0.1152	0.1527	1.69	(0.26)	2.23	(0.02)
oe96	0.0609	0.1050	0.1806	1.73	(0.30)	2.97	(0.01)
oe97	0.0606	0.0922	0.1512	1.52	(0.45)	2.50	(0.03)
oe98	0.0407	0.0796	0.1821	1.96	(0.29)	4.47	(0.01)
oe99	0.0070	0.0111	0.0603	1.58	(0.49)	8.59	(<0.01)
oe100	0.0032	0.0383	0.0939	12.04	(0.13)	29.50	(<0.01)
oe101	0.0138	0.0204	0.0672	1.47	(0.53)	4.86	(0.01)
oe102	0.0080	0.0480	0.0832	5.99	(0.04)	10.38	(<0.01)
oe103	0.0041	0.0713	0.2029	17.25	(<0.01)	49.06	(0.01)
oe104	0.0035	0.0282	0.0660	8.04	(0.03)	18.79	(<0.01)
oe105	0.0028	0.0123	0.1023	4.32	(0.21)	36.03	(0.01)
oe106	0.0812	0.0845	0.1739	1.04	(0.86)	2.14	(0.02)

Table A2 Toxicoproteomics experiment with Ethanol treated zebrafish embryos: Spot IDs. mean values and statistical analysis of all significantly repressed proteins after 2 % ethanol exposure and also their values after 0.5 % ethanol treatment: background shading indicates spots with a significant ($p<0.05$) at least two-fold difference in abundance after also 0.5 % ethanol treatment

spot name	*mean value controls*	*mean value 0.5 % ethanol*	*mean value 2 % ethanol*	*ratio 0.5% /control*	*(significance, t-test result)*	*Ratio 2%/ control*	*(significance, t-test result)*
rp 2	0.1176	0.1244	0.0180	1.06	(0.82)	-6.52	(0.01)
rp 3	0.0467	0.0455	0.0002	-1.03	(0.96)	-197.25	(0.05)
rp 4	0.1092	0.1217	0.0444	1.11	(0.42)	-2.46	(0.01)
rp 5	0.0939	0.0438	0.0084	-2.14	(0.08)	-11.21	(0.01)
rp 6	0.0975	0.0659	0.0328	-1.48	(0.04)	-2.97	(<0.01)
rp 7	0.1092	0.0635	0.0356	-1.72	(0.05)	-3.07	(0.03)
rp 8	0.1467	0.1610	0.0492	1.10	(0.45)	-2.98	(<0.01)
rp 9	0.1052	0.1129	0.0174	1.07	(0.72)	-6.05	(<0.01)
rp 10	0.1046	0.0882	0.0517	-1.19	(0.46)	-2.02	(0.04)
rp 11	0.1553	0.0899	0.0565	-1.73	(0.09)	-2.75	(<0.01)
rp13	0.1575	0.0311	0.0181	-5.07	(<0.01)	-8.71	(<0.01)
rp14	0.1705	0.0626	0.0136	-2.72	(0.05)	-12.55	(<0.01)
rp16	0.1521	0.1058	0.0640	-1.44	(0.04)	-2.38	(<0.01)
rp17	0.1367	0.1052	0.0539	-1.30	(0.34)	-2.54	(0.03)
rp19	0.1429	0.1261	0.0661	-1.13	(0.63)	-2.16	(0.04)
rp20	0.1359	0.1559	0.0510	1.15	(0.63)	-2.67	(0.05)
rp21	0.1659	0.1805	0.0616	1.09	(0.77)	-2.69	(0.03)
rp22	0.1657	0.0494	0.0565	-3.36	(0.06)	-2.93	(0.02)
rp23	0.1833	0.1598	0.0879	-1.15	(0.60)	-2.09	(0.02)
rp24	0.1634	0.1415	0.0573	-1.15	(0.78)	-2.85	(0.02)
rp25	0.2130	0.1687	0.0740	-1.26	(0.32)	-2.88	(0.01)
rp26	0.2018	0.1776	0.0924	-1.14	(0.43)	-2.19	(0.01)
rp27	0.1973	0.1718	0.0641	-1.15	(0.31)	-3.08	(<0.01)
rp28	0.1729	0.1276	0.0444	-1.35	(0.48)	-3.89	(0.02)
rp29	0.3362	0.3722	0.0947	1.11	(0.72)	-3.55	(0.04)
rp30	0.2673	0.3270	0.1300	1.22	(0.18)	-2.06	(0.01)
rp31	0.2711	0.2356	0.0368	-1.15	(0.31)	-7.37	(<0.01)
rp32	0.2371	0.2033	0.0878	-1.17	(0.45)	-2.70	(<0.01)
rp33	0.2782	0.1853	0.0914	-1.50	(0.18)	-3.04	(<0.01)
rp34	0.1992	0.2144	0.0259	1.08	(0.84)	-7.69	(0.02)
rp35	0.2341	0.1675	0.0697	-1.40	(0.34)	-3.36	(0.02)
rp36	0.3262	0.3232	0.1346	-1.01	(0.92)	-2.42	(<0.01)
rp37	0.2553	0.1962	0.0515	-1.30	(0.52)	-4.96	(0.01)
rp38	0.4095	0.2459	0.0996	-1.67	(0.09)	-4.11	(<0.01)
rp39	0.3810	0.3655	0.1741	-1.04	(0.87)	-2.19	(0.02)
rp40	0.3609	0.4278	0.1686	1.19	(0.14)	-2.14	(<0.01)
rp41	0.4779	0.3152	0.1930	-1.52	(0.11)	-2.48	(<0.01)
rp43	0.5096	0.3995	0.2084	-1.28	(0.48)	-2.45	(<0.01)
rp44	0.5804	0.6819	0.1036	1.17	(0.17)	-5.60	(<0.01)
rp45	0.4594	0.4416	0.0435	-1.04	(0.86)	-10.57	(<0.01)
rp46	0.4841	0.4667	0.1114	-1.04	(0.82)	-4.35	(<0.01)
rp47	0.5461	0.5070	0.2444	-1.08	(0.78)	-2.23	(0.04)
rp48	0.4830	0.4758	0.2056	-1.02	(0.95)	-2.35	(0.03)
rp49	0.5069	0.4395	0.1977	-1.15	(0.59)	-2.56	(0.01)
rp50	0.6921	0.6069	0.1087	-1.14	(0.34)	-6.37	(<0.01)
rp51	0.6789	0.5819	0.3342	-1.17	(0.38)	-2.03	(0.01)
rp52	0.7012	0.7337	0.2441	1.05	(0.77)	-2.87	(0.01)
rp53	0.8155	0.8388	0.3464	1.03	(0.84)	-2.35	(<0.01)
rp54	0.8667	0.8235	0.2705	-1.05	(0.62)	-3.20	(<0.01)
rp55	0.8787	0.8614	0.4265	-1.02	(0.88)	-2.06	(0.01)
rp56	1.0287	0.7520	0.3229	-1.37	(0.02)	-3.19	(<0.01)
rp57	1.2830	1.1276	0.5666	-1.14	(0.53)	-2.26	(<0.01)
rp58	1.0532	1.0436	0.3003	-1.01	(0.93)	-3.51	(<0.01)

rp59	1.2146	1.3315	0.3966	1.10	(0.48)	-3.06	(<0.01)
rp60	1.1053	1.1681	0.3754	1.06	(0.79)	-2.94	(<0.01)
rp62	1.3955	1.1765	0.5611	-1.19	(0.25)	-2.49	(<0.01)
rp63	1.6915	1.3361	0.8095	-1.27	(0.18)	-2.09	(<0.01)
rp64	0.2911	0.2865	0.1472	-1.02	(0.91)	-1.98	(0.02)
rp66	0.1332	0.0896	0.0715	-1.49	(0.17)	-1.86	(<0.01)
rp67	0.0568	0.0265	0.0220	-2.14	(0.05)	-2.58	(0.01)
rp68	0.0567	0.0153	0.0256	-3.72	(0.07)	-2.22	(0.05)
rp69	0.1380	0.0826	0.0225	-1.67	(0.24)	-6.13	(<0.01)
rp70	0.4639	0.4286	0.0557	-1.08	(0.77)	-8.33	(<0.01)
rp71	0.1048	0.0431	0.0289	-2.43	(0.19)	-3.63	(0.02)
rp72	0.1432	0.2150	0.0154	1.50	(0.09)	-9.33	(<0.01)
rp73	0.1756	0.1199	0.0232	-1.46	(0.31)	-7.58	(<0.01)
rp74	0.2212	0.1654	0.0253	-1.34	(0.26)	-8.73	(<0.01)
rp75	0.1477	0.0883	0.0360	-1.67	(0.25)	-4.10	(<0.01)
rp76	0.3067	0.2106	0.0506	-1.46	(0.33)	-6.07	(<0.01)
rp77	0.2121	0.1780	0.0892	-1.19	(0.29)	-2.38	(<0.01)
rp78	0.0446	0.0239	0.0057	-1.87	(0.10)	-7.79	(0.01)
rp79	0.1570	0.0715	0.0668	-2.20	(0.06)	-2.35	(<0.01)
rp80	0.0661	0.0382	0.0121	-1.73	(<0.01)	-5.46	(<0.01)
rp81	0.0969	0.0633	0.0281	-1.53	(0.33)	-3.45	(0.02)

Table B Toxicoproteomics experiment with Rotenone treated zebrafish larvae. Spot IDs, mean values, fold change ratio related to controls (ratio) and Student's t-test results for overexpressed and repressed proteins after EC_{10} rotenone exposure are shown.

Spot NAME	*Mean C*	*RSD [%]*	*Mean EC_{10}*	*RSD [%]*	*ratio*	*t-Test*	*Spot NAME*	*Mean C*	*RSD [%]*	*Mean EC_{10}*	*RSD [%]*	*ratio*	*t-Test*
R001o	0.19	35.1	0.38	11.6	2.0	97.4	***R036o***	0.13	32.3	0.42	15.4	3.3	99.4
R002o	0.01	24.5	0.03	16.8	2.1	97.6	***R037o***	0.27	45.4	0.91	8.7	3.3	99.6
R003o	0.65	21.7	1.37	19.5	2.1	97.1	***R038o***	0.02	65.4	0.08	11.1	3.5	99.0
R004o	0.57	46.8	1.21	6.9	2.1	96.8	***R039o***	0.13	29.5	0.44	23.7	3.5	98.4
R005o	0.08	24.6	0.16	9.5	2.1	99.2	***R040o***	0.05	21.6	0.18	30.3	3.5	97.0
R006o	0.17	15.2	0.36	9.4	2.1	99.7	***R041o***	0.09	44.3	0.38	18.7	4.1	99.2
R007o	0.20	12.4	0.43	5.5	2.2	99.9	***R042o***	0.02	49.3	0.06	10.8	4.1	99.7
R008o	0.42	9.5	0.93	6.3	2.2	99.9	***R043o***	0.00	67.9	0.02	32.1	4.2	96.0
R009o	0.18	11.9	0.40	20.2	2.2	98.0	***R044o***	0.12	39.2	0.52	11.4	4.3	99.8
R010o	0.07	35.3	0.15	20.6	2.2	96.2	***R045o***	0.07	39.6	0.31	5.6	4.3	99.9
R011o	0.27	20.4	0.63	12.2	2.3	99.4	***R046o***	0.01	70.8	0.06	9.1	4.5	99.6
R012o	0.20	10.9	0.45	12.7	2.3	99.6	***R047o***	0.04	11.5	0.17	19.7	4.6	99.5
R013o	0.19	29.3	0.45	25.1	2.3	95.5	***R048o***	0.03	59.4	0.14	13.1	4.7	99.6
R014o	0.15	12.7	0.36	11.9	2.3	99.6	***R049o***	0.06	39.6	0.29	19.3	4.8	99.4
R015o	0.10	58.2	0.23	8.7	2.3	96.3	***R050o***	0.02	50.7	0.09	4.0	4.8	99.9
R016o	0.16	19.4	0.38	13.9	2.4	99.3	***R051o***	0.03	30.1	0.16	36.4	4.9	96.2
R017o	0.03	40.0	0.07	4.1	2.4	99.1	***R052o***	0.03	102.5	0.16	23.7	5.0	97.8
R018o	0.04	2.6	0.10	16.8	2.4	99.2	***R053o***	0.03	58.1	0.19	33.6	5.7	97.1
R019o	0.53	7.2	1.30	19.1	2.5	98.8	***R054o***	0.04	21.5	0.22	24.7	5.7	99.0
R020o	0.03	14.8	0.08	15.1	2.5	99.4	***R055o***	0.09	55.9	0.51	11.3	5.7	99.9
R021o	0.06	58.4	0.15	6.2	2.5	97.6	***R056o***	0.03	56.7	0.16	26.3	5.9	98.6
R022o	0.15	21.9	0.39	29.9	2.6	95.1	***R057o***	0.12	70.4	0.72	3.9	5.9	99.9
R023o	0.26	30.5	0.67	9.8	2.6	99.5	***R058o***	0.00	141.4	0.01	23.0	5.9	97.6
R024o	0.08	59.8	0.21	4.6	2.7	98.2	***R059o***	0.02	30.6	0.11	39.7	6.0	95.8
R025o	0.02	11.5	0.05	13.0	2.7	99.7	***R060o***	0.12	29.6	0.73	21.8	6.1	99.4
R026o	0.01	35.8	0.03	12.0	2.8	99.3	***R061o***	0.02	52.6	0.12	34.0	6.4	97.3
R027o	0.08	37.9	0.23	13.4	2.8	99.1	***R062o***	0.03	92.1	0.16	22.7	6.4	98.9
R028o	0.03	27.3	0.10	20.5	2.8	98.4	***R063o***	0.05	34.3	0.32	9.2	6.4	100.0
R029o	0.01	34.8	0.03	30.4	2.9	95.2	***R064o***	0.04	72.9	0.24	18.6	6.5	99.5
R030o	0.30	70.1	0.87	13.4	2.9	97.1	***R065o***	0.01	87.6	0.04	40.3	6.7	95.3
R031o	0.03	17.8	0.09	8.0	2.9	99.9	***R066o***	0.00	87.2	0.02	24.0	6.7	98.8
R032o	0.05	69.3	0.16	19.5	3.0	96.5	***R067o***	0.02	57.0	0.11	35.1	6.8	97.1
R033o	0.05	34.6	0.14	26.7	3.0	96.9	***R068o***	0.06	67.6	0.44	9.1	7.3	99.9
R034o	0.08	49.4	0.24	23.2	3.1	97.2	***R069o***	0.08	58.8	0.58	14.2	7.5	99.8
R035o	0.11	48.6	0.35	1.5	3.2	99.7	***R070o***	0.01	28.0	0.10	15.3	7.9	99.9
R071o	0.11	38.4	0.98	9.4	8.6	100.0	***R113o***	0.01	85.0	0.95	10.5	134.9	100.0
R072o	0.07	14.4	0.67	19.7	9.0	99.7	***R114o***	0.00	141.4	0.11	17.0	652.5	99.9
R073o	0.01	72.2	0.10	6.8	9.1	100.0	***R115o***	0.00	141.4	0.07	49.1	950.0	95.5
R074o	0.01	81.9	0.11	37.8	9.1	96.8	***R116o***	0.02	24.2	0.00	141.4	0.0	99.6
R075o	0.08	34.7	0.73	9.3	9.2	100.0	***R117r***	0.04	18.2	0.00	141.4	0.0	99.8
R076o	0.03	78.9	0.26	39.6	9.3	96.4	***R118r***	0.55	43.1	0.01	141.4	-100	96.8

Spot NAME	Mean C	RSD [%]	Mean EC_{10}	RSD [%]	ratio	t-Test	Spot NAME	Mean C	RSD [%]	Mean EC_{10}	RSD [%]	ratio	t-Test
R077o	0.02	114.6	0.21	5.9	9.6	99.9	***R119r***	0.08	41.6	0.00	141.4	-100	97.2
R077o	0.02	114.6	0.21	5.9	9.6	99.9	***R120r***	0.10	22.1	0.00	93.2	-100	99.7
R078o	0.05	41.9	0.52	24.2	9.7	99.3	***R121r***	0.01	35.8	0.00	141.4	-100	98.2
R079o	0.01	82.0	0.11	41.6	10.3	96.1	***R122r***	0.06	18.3	0.00	97.7	-10	99.8
R080o	0.01	95.0	0.07	7.0	10.7	100.0	***R123r***	0.29	15.1	0.02	31.6	-10	99.9
R081o	0.08	57.9	0.91	2.9	10.9	100.0	***R124r***	0.08	23.6	0.01	78.5	-10	99.4
R082o	0.00	59.2	0.01	35.2	11.5	97.8	***R125r***	0.15	30.2	0.01	90.0	-10	98.5
R083o	0.03	54.0	0.31	6.0	11.7	100.0	***R126r***	0.14	36.2	0.01	74.2	-10	97.5
R084o	0.01	14.5	0.11	8.5	12.1	100.0	***R127r***	0.16	20.4	0.01	68.5	-10	99.6
R085o	0.03	123.3	0.42	12.3	12.3	99.9	***R128r***	0.06	31.2	0.01	70.0	-10	98.3
R086o	0.03	64.4	0.41	3.0	12.9	100.0	***R129r***	0.04	4.0	0.00	137.9	-10	99.9
R087o	0.03	100.1	0.42	9.7	13.5	100.0	***R130r***	0.89	23.4	0.10	141.4	-10	98.9
R088o	0.08	64.0	1.04	11.8	13.6	100.0	***R131r***	0.09	37.5	0.01	96.8	-10	96.6
R089o	0.02	59.6	0.26	42.5	13.8	96.3	***R132r***	0.23	21.2	0.03	61.3	-10	99.4
R090o	0.03	34.2	0.42	30.4	14.0	98.7	***R133r***	0.05	20.9	0.01	45.0	-10	99.5
R091o	0.01	98.3	0.15	30.2	14.3	98.7	***R134r***	0.05	19.0	0.01	87.8	-10	99.4
R092o	0.00	86.7	0.03	16.5	14.9	99.8	***R135r***	0.08	17.8	0.01	85.1	-10	99.5
R093o	0.03	37.8	0.45	15.1	15.2	99.9	***R136r***	0.19	18.0	0.03	89.1	-10	99.4
R094o	0.02	74.8	0.32	23.7	16.0	99.5	***R137r***	0.53	20.5	0.08	89.0	-5	99.1
R095o	0.01	85.5	0.23	34.8	17.4	98.1	***R138r***	0.47	10.6	0.08	63.2	-5	99.9
R096o	0.03	48.1	0.57	12.1	18.1	100.0	***R139r***	0.24	36.1	0.04	73.0	-5	96.2
R097o	0.01	51.3	0.25	16.7	18.2	99.9	***R140r***	0.11	27.8	0.02	64.0	-5	98.2
R098o	0.01	106.3	0.13	32.6	18.2	98.4	***R141r***	0.04	15.6	0.01	131.8	-5	98.3
R099o	0.01	54.3	0.10	42.5	19.0	96.5	***R142r***	0.17	7.0	0.03	42.6	-5	100.0
R100o	0.00	141.4	0.01	40.6	19.3	96.9	***R143r***	0.14	6.9	0.03	24.9	-5	100.0
R101o	0.03	30.5	0.51	6.4	20.3	100.0	***R144r***	0.20	24.4	0.04	52.0	-5	98.7
R102o	0.01	81.6	0.21	16.3	20.4	99.9	***R145r***	0.02	27.9	0.00	72.2	-5	97.8
R103o	0.00	97.9	0.10	20.4	23.7	99.7	***R146r***	0.06	10.6	0.01	76.7	-5	99.6
R104o	0.00	131.9	0.04	45.5	25.7	95.9	***R147r***	0.11	23.7	0.02	67.5	-5	98.3
R105o	0.01	89.8	0.15	48.0	27.7	95.3	***R148r***	0.34	9.3	0.08	44.4	-5	99.9
R106o	0.00	141.4	0.02	41.1	31.4	97.0	***R149r***	0.37	20.2	0.09	40.0	-5	99.1
R107o	0.02	126.8	0.55	18.3	34.8	99.8	***R150r***	0.21	12.3	0.05	15.6	-5	99.9
R108o	0.00	123.3	0.08	40.0	40.5	97.4	***R151r***	0.07	15.0	0.02	41.3	-3.3	99.6
R109o	0.01	135.5	0.23	13.2	40.6	99.9	***R152r***	0.12	8.0	0.03	59.4	-3.3	99.7
R110o	0.00	84.3	0.03	45.4	44.0	96.2	***R153r***	0.12	21.9	0.03	43.9	-3.3	98.7
R111o	0.01	79.6	0.47	13.1	47.3	100.0	***R154r***	1.34	11.5	0.35	49.2	-3.3	99.6
R112o	0.01	119.5	0.45	27.5	84.0	99.3	***R155r***	0.34	12.4	0.09	48.5	-3.3	99.6
R156r	0.28	23.5	0.07	41.4	-3.33	98.4	***R182r***	0.10	19.1	0.04	23.6	-2.5	98.6
R157r	0.18	27.8	0.05	30.3	-3.33	97.7	***R183r***	0.21	2.2	0.08	58.1	-2.5	98.3
R158r	0.27	20.6	0.07	33.0	-3.33	99.0	***R184r***	0.07	17.9	0.03	20.8	-2.5	98.8
R159r	0.13	14.9	0.06	41.8	-2	95.0	***R185r***	0.99	11.7	0.41	23.5	-2.5	99.5
R160r	0.23	3.4	0.06	71.1	-3.33	99.4	***R186r***	0.22	21.3	0.09	10.0	-2.5	98.2
R161r	0.75	11.6	0.21	66.0	0.3	99.1	***R187r***	0.05	29.2	0.02	5.0	-2.5	95.1
R162r	0.22	27.2	0.06	18.8	-3.33	97.9	***R188r***	0.30	13.7	0.13	5.0	-2.5	99.6

Spot NAME	*Mean C*	*RSD [%]*	*Mean EC_{10}*	*RSD [%]*	*ratio*	*t-Test*	*Spot NAME*	*Mean C*	*RSD [%]*	*Mean EC_{10}*	*RSD [%]*	*ratio*	*t-Test*
R163r	0.24	24.7	0.07	35.4	-3.3	98.1	***R189r***	0.30	11.0	0.13	4.2	-2.5	99.8
R164r	0.52	20.8	0.15	13.6	-3.3	99.1	***R190r***	0.15	10.0	0.07	42.9	-2.5	97.9
R165r	0.13	24.4	0.04	26.8	-3.3	98.3	***R191r***	0.41	17.4	0.18	18.6	-2.5	98.5
R166r	0.07	5.0	0.02	47.9	-3.3	99.7	***R192r***	0.29	1.6	0.13	25.5	-2.5	99.8
R167r	0.28	11.8	0.09	28.2	-3.3	99.7	***R193r***	0.14	13.0	0.06	8.6	-2.5	99.6
R168r	0.29	21.7	0.09	17.7	-3.3	98.7	***R194r***	0.29	4.1	0.13	28.9	-2.5	99.5
R169r	0.21	3.4	0.07	27.8	-3.3	99.9	***R195r***	0.17	6.4	0.08	24.9	-2.5	99.6
R170r	0.09	31.0	0.03	30.1	-3.3	95.7	***R196r***	0.44	7.6	0.20	21.5	-2.5	99.7
R171r	0.52	15.7	0.17	73.5	-3.3	96.9	***R197r***	0.12	4.3	0.05	31.6	-2.5	99.3
R172r	0.89	4.8	0.30	35.0	-3.3	99.8	***R198r***	1.67	18.1	0.76	29.8	-2.5	97.2
R173r	0.06	27.1	0.02	16.6	-3.3	97.3	***R199r***	0.45	8.7	0.21	24.2	-2	99.4
R174r	0.22	16.4	0.07	11.9	-3.3	99.5	***R200r***	0.12	11.8	0.06	8.2	-2	99.6
R175r	0.22	26.4	0.08	50.6	-2.5	95.5	***R201r***	0.19	4.8	0.09	33.4	-2	98.9
R176r	0.25	9.8	0.09	40.2	-2.5	99.4	***R202r***	0.35	5.8	0.17	27.1	-2	99.3
R177r	0.59	29.1	0.21	30.4	-2.5	95.7	***R203r***	0.21	15.7	0.11	24.6	-2	97.7
R178r	0.28	15.8	0.10	18.5	-2.5	99.4	***R204r***	0.54	12.4	0.27	19.4	-2	98.9
R179r	0.47	23.8	0.17	24.7	-2.5	97.7	***R205r***	0.39	10.2	0.19	6.8	-2	99.7
R180r	0.56	12.3	0.21	32.9	-2.5	99.4	***R206r***	0.87	10.5	0.44	14.5	-2	99.5
R181r	1.02	1.7	0.37	36.6	-2.5	99.7	***R207r***	0.87	10.5	0.44	14.5	-2	99.5

Table C Toxicoproteomics experiment with DNOC treated zebrafish larvae. Spot IDs. mean values, fold change ratio related to controls and Student's t-test results of overexpressed and repressed proteins after EC_{10} and EC_{40} DNOC exposure are shown.

Spot Nr.	*Mean C*	*RSD [%]*	*Mean EC_{10}*	*RSD [%]*	*Ratio*	*t-Test*	*Mean EC_{40}*	*RSD [%]*	*Ratio*	*t-Test*	*SpotID*	*changed at EC*	
1	0.02	47.5	0.02	28.0	1.18	32.10	0.05	15.7	3.0	96.3	***D1oe***		40
2	0.21	14.2	0.33	11.9	1.55	97.11	0.46	13.8	2.2	98.0	***D2oe***		40
3	0.17	34.1	0.30	14.1	1.77	93.78	0.45	15.4	2.7	96.8	***D3oe***		40
4	0.02	32.1	0.04	74.2	1.81	54.77	0.06	13.4	3.0	98.3	***D4oe***		40
5	0.04	40.6	0.08	15.7	1.85	92.55	0.11	5.1	2.6	97.5	***D5oe***		40
6	0.07	28.5	0.14	37.1	1.93	83.64	0.15	9.8	2.1	96.6	***D6o***		40
7	0.03	55.7	0.08	31.8	2.89	93.27	0.13	22.8	4.6	96.9	***D7o***		40
8	0.14	56.8	0.31	18.6	2.17	92.40	0.41	2.2	2.9	96.3	***D8o***		40
9	0.01	54.6	0.03	130.0	2.46	44.11	0.05	8.7	4.2	98.9	***D9o***		40
10	0.01	82.5	0.05	38.4	3.33	90.38	0.07	14.3	4.7	97.4	***D10o***		40
11	0.00	109.1	0.08	73.6	16.64	85.38	0.05	30.5	10.9	96.6	***D11o***		40
12	0.00	88.1	0.03	58.8	27.01	91.83	0.03	13.0	25.4	99.7	***D12o***		40
13	0.03	77.4	0.10	61.3	3.48	80.24	0.19	29.9	6.5	95.8	***D130***		40
14	0.00	115.3	0.02	62.3	33.08	90.71	0.04	17.1	64.7	99.5	***D14o***		40
15	0.01	37.8	0.05	51.8	3.76	87.93	0.05	6.2	3.8	99.4	***D15o***		40
16	0.00	141.4	0.01	101.4	349.62	76.32	0.07	2.6	3667.3	100.0	***D16o***		40
17	0.03	79.3	0.14	25.2	3.98	96.99	0.13	27.2	3.9	92.6	***D17o***		40
18	0.00	141.4	0.01	108.2	410.52	73.76	0.03	33.9	1522.7	97.1	***D18o***		40
19	0.02	78.0	0.08	49.9	4.26	88.86	0.08	11.8	4.3	97.4	***D19o***		40
20	0.01	55.2	0.03	44.7	4.75	92.71	0.02	3.4	4.1	99.1	***D20o***		40
21	0.05	29.2	0.26	41.4	5.01	94.62	0.32	0.6	6.2	100.0	***D21o***		40
22	0.01	87.5	0.04	85.8	5.30	74.05	0.07	28.6	10.7	97.2	***D22o***		40
23	0.01	109.1	0.03	70.8	5.33	80.60	0.04	0.6	7.0	99.1	***D23o***		40
24	0.00	141.4	0.01	51.7	5.77	88.96	0.02	19.4	8.2	97.4	***D24o***		40
25	0.01	76.5	0.03	54.2	6.61	90.38	0.05	33.2	10.4	96.1	***D25o***		40
26	0.01	89.8	0.06	51.7	7.95	92.04	0.09	35.2	12.0	95.7	***D26o***		40
27	0.00	141.4	0.02	53.7	8.25	90.80	0.04	28.0	16.2	97.5	***D27o***		40
28	0.01	101.3	0.10	46.7	8.50	93.94	0.15	13.5	12.3	99.5	***D28o***		40
29	0.00	101.5	0.02	113.2	9.08	66.94	0.01	13.3	6.1	98.1	***D29o***		40
30	0.00	94.8	0.04	55.6	11.47	91.68	0.06	17.1	17.1	99.4	***D30o***		40
31	0.00	135.1	0.02	68.1	12.85	86.87	0.02	17.3	16.0	99.2	***D31o***		40
32	0.01	69.8	0.09	55.1	15.40	92.49	0.09	3.5	14.2	100.0	***D32o***		40
33	0.00	60.3	0.07	52.6	16.47	93.45	0.05	24.7	13.6	98.4	***D33o***		40
34	0.01	119.7	0.06	26.6	10.81	98.88	0.05	28.5	9.3	96.5	***D34o***	10	40
35	0.01	141.4	0.07	33.1	11.98	97.90	0.06	2.6	10.1	99.4	***D35o***	10	40
36	0.00	141.4	0.02	32.9	12.40	97.99	0.04	38.8	22.6	95.2	***D36o***	10	40
37	0.00	82.8	0.09	11.6	26.09	99.97	0.09	28.4	26.1	98.0	***D37o***	10	40
38	0.00	139.6	0.09	15.7	41.79	99.90	0.05	2.1	22.2	99.9	***D38o***	10	40
39	0.15	23.6	0.32	6.0	2.10	99.57	0.39	4.3	2.5	99.3	***D39o***	10	40
40	0.04	33.6	0.13	18.5	3.11	98.90	0.14	0.9	3.4	99.5	***D40o***	10	

Spot Nr.	*Mean C*	*RSD [%]*	*Mean EC_{10}*	*RSD [%]*	*Ratio*	*t-Test*	*Mean EC_{40}*	*RSD [%]*	*Ratio*	*t-Test*	*SpotID*	*changed at EC*	
41	0.06	20.0	0.18	10.0	3.13	99.87	0.17	3.0	2.8	99.8	***D41o***	10	40
42	0.11	41.0	0.40	4.0	3.59	99.89	0.47	20.6	4.2	97.7	***D42o***	10	40
43	0.08	50.2	0.32	17.0	3.81	99.17	0.27	0.8	3.2	98.3	***D43o***	10	40
44	0.08	98.2	0.34	14.9	4.17	98.18	0.36	3.1	4.4	96.8	***D44o***	10	40
45	0.05	60.2	0.23	24.1	4.21	98.17	0.22	4.3	4.1	98.8	***D45o***	10	40
46	0.02	57.5	0.08	30.2	4.61	97.26	0.09	4.8	5.1	99.5	***D46o***	10	40
47	0.03	59.2	0.15	35.0	4.81	96.08	0.15	26.2	4.7	96.0	***D47o***	10	40
48	0.06	76.4	0.34	21.8	6.09	99.07	0.38	8.6	6.8	99.4	***D48o***	10	40
49	0.04	64.8	0.27	26.0	6.91	98.81	0.22	13.8	5.7	98.9	***D49o***	10	40
50	0.01	6.5	0.11	19.1	8.04	99.71	0.10	15.8	7.5	99.5	***D50o***	10	40
51	0.01	67.7	0.09	27.6	10.30	98.92	0.08	29.0	9.4	97.1	***D51o***	10	40
52	0.01	62.8	0.12	25.1	10.68	99.23	0.05	10.1	4.4	98.6	***D52o***	10	40
53	0.02	85.9	0.06	23.5	3.41	95.60	0.04	88.7	1.9	41.0	***D53o***	10	40
54	0.01	86.0	0.03	1.2	3.43	96.76	0.01	14.0	-1.43	24.9	***D54o***	10	
55	0.04	76.7	0.16	26.3	3.67	96.26	0.12	7.1	2.7	90.8	***D55o***	10	
56	0.02	99.0	0.10	18.8	3.91	97.11	0.04	84.8	1.7	33.5	***D56o***	10	
57	0.05	67.3	0.20	7.5	4.03	99.56	0.11	32.2	2.1	75.1	***D57o***	10	
58	0.13	34.4	0.27	8.1	2.04	98.15	0.19	40.3	1.4	50.3	***D58o***	10	
59	0.00	95.0	0.02	43.8	32.69	96.45	0.01	39.5	14.2	94.6	***D59o***	10	
60	0.03	32.8	0.06	13.9	2.07	97.41	0.01	31.5	-2.5	85.5	***D60o***	10	
61	0.03	31.3	0.09	5.7	3.24	99.90	0.05	57.3	1.8	58.5	***D61o***	10	
62	0.20	32.8	0.43	6.9	2.18	99.01	0.17	67.5	-1.1	19.0	***D62o***	10	
63	0.22	11.7	0.48	19.4	2.19	98.14	0.25	4.8	1.1	63.7	***D63o***	10	
64	0.17	41.8	0.37	15.5	2.25	96.88	0.33	13.6	2.0	89.4	***D64o***	10	
65	0.03	42.0	0.07	11.1	2.26	97.80	0.02	35.8	-1.43	49.9	***D65o***	10	
66	0.16	44.7	0.36	12.3	2.30	97.42	0.09	14.1	-1.66	61.9	***D66o***	10	
67	0.39	36.7	0.92	23.9	2.36	95.38	0.63	0.7	1.6	83.7	***D67o***	10	
68	0.07	47.8	0.17	3.6	2.36	98.34	0.23	5.0	3.1	98.2	***D68o***	10	
69	0.08	51.6	0.25	31.5	3.29	95.10	0.11	9.6	1.4	55.5	***D69o***	10	
70	0.01	43.9	0.04	14.5	2.75	98.59	0.02	0.3	1.1	11.7	***D70o***	10	
71	0.11	74.5	0.29	12.0	2.75	96.13	0.42	8.4	3.9	97.2	***D71o***	10	
72	0.04	52.1	0.12	20.0	2.85	97.23	0.05	13.9	1.3	40.5	***D72o***	10	
73	0.02	73.3	0.07	18.0	2.86	95.71	0.05	33.9	2.1	72.4	***D73o***	10	
74	0.10	53.8	0.30	5.7	2.90	99.12	0.12	51.2	1.2	19.2	***D74o***	10	
75	0.04	73.5	0.12	19.9	3.06	96.20	0.12	61.3	3.1	72.8	***D75o***	10	
76	0.08	90.2	0.25	15.7	3.07	95.41	0.21	10.6	2.5	83.3	***D76o***	10	
77	0.02	53.5	0.07	23.9	3.07	96.79	0.02	51.4	1.0	0.2	***D77o***	10	
78	0.02	82.2	0.06	20.7	3.12	95.46	0.01	36.4	-2	46.5	***D78o***	10	
79	0.04	19.1	0.13	12.0	3.16	99.80	0.07	35.2	1.7	77.0	***D79o***	10	
80	0.10	60.0	0.44	20.9	4.32	98.77	0.17	62.5	1.6	45.8	***D80o***	10	
81	0.07	49.9	0.34	13.8	4.61	99.68	0.20	28.6	2.6	89.0	***D81o***	10	

Spot Nr.	*Mean C*	*RSD [%]*	*Mean EC_{10}*	*RSD [%]*	*Ratio*	*t-Test*	*Mean EC_{40}*	*RSD [%]*	*Ratio*	*t-Test*	*SpotID*	*changed at EC*	
82	0.04	67.9	0.17	33.6	4.62	96.09	0.05	43.4	1.4	34.9	***D82o***	10	
83	0.04	32.9	0.19	25.4	5.26	98.82	0.17	44.1	4.9	90.2	***D83o***	10	
84	0.02	123.8	0.10	24.5	5.30	97.24	0.01	83.3	-2.5	35.9	***D84o***	10	
85	0.07	62.5	0.35	23.5	5.31	98.80	0.18	22.9	2.7	89.7	***D85o***	10	
86	0.01	98.2	0.08	29.2	6.02	97.57	0.04	18.6	3.0	85.9	***D86o***	10	
87	0.01	85.1	0.04	28.1	7.57	98.46	0.03	95.2	4.6	63.9	***D87o***	10	
88	0.00	99.4	0.04	17.1	10.81	99.73	0.04	67.9	11.4	82.7	***D88o***	10	
89	0.00	117.9	0.04	16.7	32.41	99.87	0.09	65.4	68.5	86.4	***D89o***	10	
90	0.32	10.5	0.25	7.4	-1.28	94.32	0.14	42.0	-2.5	95.8	***D90r***		40
91	0.68	12.3	0.36	7.2	-1.88	99.32	0.27	38.2	-2.5	96.6	***D91r***		40
92	0.53	14.4	0.29	8.5	-1.78	98.55	0.03	72.0	-10	99.4	***D92r***		40
93	0.18	18.1	0.11	21.2	-1.58	92.15	0.07	5.1	-2.5	96.3	***D93r***		40
94	0.19	22.8	0.09	37.8	-2.04	92.89	0.04	42.5	-5	96.0	***D94r***		40
95	0.02	30.5	0.00	86.4	-100	99.01	0.00		-100	96.3	***D95r***	10	40
96	0.07	22.4	0.02	75.2	-3.7	97.28	0.01	78.9	-5	96.7	***D96r***	10	40
97	0.11	14.1	0.04	76.1	0.32	97.28	0.03	27.2	-5	98.8	***D97r***	10	40
98	0.10	6.0	0.03	59.3	-3.12	98.90	0.05	37.5	-2	96.7	***D98r***	10	40
99	0.06	16.8	0.02	71.3	-2.7	95.52	0.02	43.3	-3.3	95.8	***D99r***	10	40
100	0.07	21.9	0.03	48.5	-2.56	95.92	0.02	35.6	-3.3	95.4	***D100r***	10	40
101	0.23	12.2	0.11	15.9	-2.08	99.30	0.11	4.2	-2	98.1	***D101r***	10	40
102	0.03	36.8	0.00	65.4	-20	97.78	0.00	100.0	-10	92.0	***D102r***	10	40
103	0.04	21.6	0.01	71.1	-5.89	99.06	0.03	100.0	-1.25	27.0	***D103r***	10	
104	0.07	39.8	0.01	3.7	-5.55	95.60	0.04	39.4	-1.66	65.5	***D104r***	10	
105	0.26	27.3	0.07	57.9	-4	97.33	0.05	87.6	-5	93.6	***D105r***	10	
106	0.23	27.5	0.07	59.9	-3.22	95.90	0.06	56.8	-5	93.2	***D106r***	10	
107	0.18	29.4	0.06	32.7	-2.86	95.69	0.09	64.3	-2	74.9	***D107r***	10	
108	0.07	18.7	0.03	41.9	-2.56	97.42	0.03	30.9	-2	92.7	***D108r***	10	
109	0.20	19.8	0.09	42.1	-2.38	96.30	0.19	0.4	-1.1	34.3	***D109r***	10	
110	0.20	9.2	0.09	27.5	-2.22	99.25	0.15	27.1	-1.25	70.9	***D110r***	10	
111	0.12	19.4	0.06	30.5	-2.13	96.47	0.08	19.2	-1.66	82.3	***D111r***	10	
112	0.51	17.4	0.25	30.6	-2.08	96.82	0.24	26.1	-1.5	93.7	***D112r***	10	
113	0.32	9.3	0.15	19.8	-2.04	99.43	0.16	24.7	-2	96.9	***D113r***	10	
114	0.09	15.9	0.04	36.1	-2.04	96.13	0.09	7.8	1.0	0.4	***D114r***	10	

Table D Toxicoproteomics experiment with Diclofenac treated zebrafish larvae. Spot IDs. mean values, fold change ratio related to controls and Student's t-test results of all overexpressed and repressed proteins after EC_{10} and EC_{01} diclofenac exposure are shown.

Nr	*Mean controls*	*RSD [%]*	*Mean EC_1*	*RSD [%]*	*ratio*	*t-Test*	*Mean EC_{10}*	*RSD [%]*	*ratio*	*t-Test*	*Spot ID*	*Changed at EC*	
1	0.02	53.7	0.04	7.4	2.1	95.1	0.03	11.2	1.6	76.0	***Dic1o***	1	
2	0.11	24.7	0.25	15.4	2.1	98.3	0.18	18.2	1.6	90.9	***Dic2o***	1	
3	0.03	8.1	0.07	26.4	2.2	95.5	0.06	51.5	2.0	74.7	***Dic3o***	1	
4	0.02	75.9	0.07	14.9	2.8	95.8	0.08	70.2	3.2	74.4	***Dic4o***	1	
5	0.04	62.3	0.13	23.8	2.9	95.6	0.09	56.8	1.9	65.0	***Dic5o***	1	
6	0.02	70.1	0.07	18.9	2.9	96.2	0.03	36.2	1.3	35.5	***Dic6o***	1	
7	0.01	36.1	0.04	33.3	3.3	95.1	0.04	40.6	3.4	92.3	***Dic7o***	1	
8	0.01	72.4	0.03	13.2	3.5	98.6	0.03	55.7	2.8	78.8	***Dic8o***	1	
9	0.00	82.2	0.01	20.0	4.2	98.2	0.04	62.4	10.4	88.8	***Dic9o***	1	
10	0.01	76.9	0.04	35.9	4.6	95.1	0.01	85.1	1.4	28.8	***Dic10o***	1	
11	0.13	39.8	0.33	23.5	2.5	96.0	0.32	17.6	2.4	97.4	***Dic11o***	1	10
12	0.06	21.7	0.15	22.0	2.6	97.9	0.13	1.8	2.3	99.9	***Dic12o***	1	10
13	0.04	16.4	0.11	27.4	3.0	97.1	0.14	35.8	3.7	95.4	***Dic13o***	1	10
14	0.04	60.3	0.13	16.1	3.1	98.2	0.13	12.9	3.0	98.2	***Dic14o***	1	10
15	0.04	48.9	0.11	23.1	3.2	97.5	0.13	21.8	3.7	98.5	***Dic15o***	1	10
16	0.01	70.5	0.04	26.3	3.4	95.9	0.07	9.1	5.9	99.9	***Dic16o***	1	10
17	0.02	61.6	0.10	13.8	4.6	99.5	0.10	5.1	4.7	99.9	***Dic17o***	1	10
18	0.00	83.1	0.05	44.7	16.5	95.8	0.06	21.6	18.3	99.6	***Dic18o***	1	10
19	0.00	77.7	0.10	30.6	23.5	98.8	0.09	9.9	21.5	100.0	***Dic19o***	1	10
20	0.00	33.3	0.09	30.7	24.3	98.8	0.13	21.8	33.8	99.7	***Dic20o***	1	10
21	0.02	68.4	0.03	31.3	1.6	60.1	0.06	10.3	4.0	99.4	***Dic21o***		10
22	0.02	19.3	0.04	10.5	1.6	97.3	0.05	23.8	2.1	95.3	***Dic22o***		10
23	0.08	28.0	0.13	11.0	1.7	95.7	0.18	13.9	2.4	98.9	***Dic23o***		10
24	0.05	13.0	0.09	19.6	1.8	95.7	0.11	12.0	2.0	99.4	***Dic24o***		10
25	0.03	51.2	0.06	7.7	1.8	91.3	0.07	13.6	2.2	95.3	***Dic25o***		10
26	0.06	38.9	0.11	34.3	1.9	82.6	0.12	10.6	2.1	97.7	***Dic26o***		10
27	0.08	26.2	0.15	20.5	1.9	94.7	0.18	17.0	2.3	98.2	***Dic27o***		10
28	0.04	20.9	0.08	6.9	2.0	99.5	0.10	9.6	2.3	99.7	***Dic28o***		10
29	0.05	19.8	0.11	44.2	2.0	80.6	0.15	9.8	2.8	99.8	***Dic29o***		10
30	0.03	31.7	0.07	25.3	2.1	93.8	0.12	26.2	3.4	97.6	***Dic30o***		10
31	0.08	35.3	0.18	33.1	2.2	89.6	0.23	25.7	2.7	96.4	***Dic31o***		10
32	0.03	52.6	0.06	27.7	2.2	90.1	0.06	12.1	2.5	97.5	***Dic32o***		10
33	0.03	43.9	0.06	51.9	2.3	77.3	0.08	25.3	2.7	95.8	***Dic33o***		10
34	0.02	68.2	0.03	11.4	2.3	93.1	0.06	17.9	4.0	98.7	***Dic34o***		10
35	0.02	73.7	0.05	3.3	2.3	93.4	0.08	27.4	3.6	96.0	***Dic35o***		10
36	0.03	16.5	0.07	73.0	2.6	69.6	0.09	28.7	3.2	97.1	***Dic36o***		10
37	0.00	136.8	0.00	72.1	2.7	63.4	0.03	28.8	36.8	99.1	***Dic37o***		10
38	0.00	9.7	0.01	124.0	3.0	50.9	0.01	21.2	3.2	98.9	***Dic38o***		10
39	0.02	66.4	0.10	47.0	4.1	90.3	0.12	21.2	5.1	99.0	***Dic39o***		10
40	0.01	90.5	0.04	55.5	4.2	85.5	0.05	24.4	5.3	98.2	***Dic40o***		10

Nr	*Mean controls*	*RSD [%]*	*Mean EC_1*	*RSD [%]*	*ratio*	*t-Test*	*Mean EC_{10}*	*RSD [%]*	*ratio*	*t-Test*	*Spot ID*	*Changed at EC*	
41	0.01	68.1	0.04	48.6	4.8	90.9	0.04	28.0	4.4	97.3	***Dic41o***		10
42	0.00	122.5	0.02	78.9	6.0	77.9	0.17	38.1	44.9	97.8	***Dic42o***		10
43	0.00	75.5	0.01	79.9	8.1	80.2	0.01	39.4	14.2	97.0	***Dic43o***		10
44	0.01	81.9	0.05	63.1	9.7	88.3	0.03	15.8	5.8	99.5	***Dic44o***		10
45	0.00	55.9	0.02	102.0	12.1	72.7	0.01	24.8	9.9	99.2	***Dic45o***		10
46	0.00	141.4	0.02	79.1	17.0	83.1	0.03	36.9	20.6	97.7	***Dic46o***		10
47	0.00	94.9	0.06	83.8	35.4	82.4	0.04	32.4	26.5	98.6	***Dic47o***		10
48	0.00	141.4	0.03	54.3	45.8	93.6	0.03	31.4	39.2	98.8	***Dic48o***		10
49	0.00		0.02	125.7	∞	67.6	0.01	11.7	∞	100.0	***Dic49o***		10
50	0.00		0.02	73.1	∞	87.5	0.02	35.4	∞	98.4	***Dic50o***		10
51	0.17	35.1	0.20	21.1	1.2	46.6	0.45	10.2	2.7	99.4	***Dic51o***		10
52	0.02	10.7	0.04	23.1	2.1	96.4	0.01	79.0	-3.3	96.6	***Dic52or***	1	10
53	0.09	11.9	0.04	50.0	0.4	97.6	0.08	53.0	-1.1	30.1	***Dic53r***	1	
54	0.12	3.4	0.06	12.2	0.5	99.9	0.06	27.9	-2	99.2	***Dic54r***	1	
55	0.03	26.4	0.02	103.0	0.5	65.4	0.00		-10	99.4	***Dic55r***		10
56	0.90	17.6	0.55	34.3	0.6	88.2	0.39	40.4	-2.5	96.8	***Dic56r***		10
57	0.12	18.8	0.08	29.5	0.7	84.9	0.05	51.4	-2.5	96.3	***Dic57r***		10
58	0.03	21.2	0.02	14.6	0.7	88.3	0.01	52.2	-5	98.8	***Dic58r***		10
59	0.04	14.7	0.02	59.7	0.7	66.1	0.01	49.4	-3.3	98.8	***Dic59r***		10
60	0.06	10.1	0.04	59.2	0.7	63.6	0.03	40.3	-2	97.4	***Dic60r***		10
61	0.19	25.4	0.13	10.2	0.7	81.3	0.09	13.8	-2	95.3	***Dic61r***		10
62	0.19	23.1	0.14	5.7	0.7	83.3	0.09	3.9	-2	97.1	***Dic62r***		10
63	0.12	19.3	0.09	8.7	0.8	83.3	0.06	38.2	-2	95.7	***Dic63r***		10
64	0.17	8.5	0.13	15.8	0.8	87.6	0.08	15.4	-2	99.7	***Dic64r***		10
65	0.39	5.5	0.32	19.2	0.8	77.4	0.19	18.1	-2	99.8	***Dic65r***		10
66	0.09	18.0	0.08	24.9	0.9	26.5	0.04	18.1	-2.5	98.4	***Dic66r***		10

Table E Comparison of all differentially expressed proteins in toxicoproteomics experiments with Rotenone. DNOC and Diclofenac treated zebrafish larvae.

Spot	*Rotenone*	*DNOC*	*Diclofenac*	*only EC_{10}*
1	R036o	D071o		DR1
2	R010o			
3	R017o			
4		D022o		
5	R194r			
6	R070o			
7			Dic40o	
8	R042o			
9	R024o			
10	R168r		Dic66r	DicR1
11	R199r			
12	R141r			
13	R201r			
14	R143r			
15	R195r			
16	R183r			
17	R169r	D108r		DR2
18	R193r			
19	R136r			
20		D107r		
21			Dic38o	
22	R078o	D032o		
23	R067o			
24	R089o			
25	R75o	D048o		DR3
26	R081o	D049o		DR4
27	R149r			
28	R196r	D112r		DR5
29	R107o	D055o	Dic19o	DicDR1
30	R021o		Dic13o	DicR2
31			Dic3o	
32		D052o		
33	R159r			
34	R186r		Dic57r	DicR3
35	R150r	D093r	Dic62r	DicR4
36	R176r			
37	R182r			
38	R172r			
39	R079o			
40	R189r	D101r	Dic64r	DicDR2
41		D111r		
42	R001o			
43		D113r		
44	R164r			
45	R154r			
46 93	R019o		Dic65r	
94	R008o			
95		D076o		

Spot	*Rotenone*	*DNOC*	*Diclofenac*	*only EC_{10}*
47	R055o			
48	R039o			
49	R206r			
50	R188r			
51	R207r			
52	R046o			
53	R016o			
54		D068o		
55		D005o		
56	R203r			
57	R025o			
58	R190r			
59	R202r			
60	R102o			
61		D063o		
62	R106o			
63	R043o			
64			Dic2o	
65		D057o		
66		D010o		
67	R090o			
68	R064o			
69		D024o	Dic9o	
70	R105o	D013o		
71	R146r			
72	R110o			
73		D016o		
74			Dic52o	
75			Dic46o	
76			Dic37o	
77		D038o		
78		D018o		
79		D014o		
80		D031o		
81			Dic63r	
82	R161r	D069o		DR6
83		D090r		
84	R185r			
85	R137r			
86	R139r		Dic61r	DicR4
87		D067o		
88	R180r			
89	R155r			
90	R131r			
91			Dic30o	
92 144	R119r	D102r		
145	R091o	D009o		DicR7
146			Dic180	

Spot	*Rotenone*	*DNOC*	*Diclofenac*	*only EC_{10}*
96	R191r	D091r		
97			Dic55r	
98	R171r			
99			Dic42o	
100	R128r			
101		D096r		
102	R004r			
103	R133r			DicDR3
104	R098r			
105	R071o	D043o	Dic12o	DicDR4
106			Dic32o	DICR5
107	R068o	D051o	Dic41o	
108	R060o	D003o	Dic23o	
109		D002o		
110	R100o			
111	R074o			DICDR5
112	R065o			
113	R037o	D042o	Dic27o	
114	R040o			
115	R147r			
116			Dic36o	
117	R170r			
118		D011o		
119	R057o			
120	R122r			DicR6
121	R054o			
122	R087o		Dic31o	
123	R163r			
124	R112o		Dic10o	
125	R026o			
126	R023o			DR7
127			Dic58r	
128	R096o	D034o		
129	R059o			
130		D047o		DicDR6
131	R113o	D021o	Dic5o	
132	R088o	D044o	Dic11o	
133	R018o			DicDR7
134	R083o	D019o		
135	R111o	D040o	Dic14o	
136	R048o			
137		D039o		
138	R086o			
139	R062o			
140		D059o		
141	R142r			
142			Dic44o	
143		D004o		
195	R077o			
196	R076o			
197		D081o		

Spot	*Rotenone*	*DNOC*	*Diclofenac*	*only EC_{10}*
147	R069o		Dic48o	
148	R032o			
149		D058o		
150			Dic8o	
151			Dic28o	
152	R056o			
153	R011o			
154	R094o			
155	R051o			
156	R047o			
157	R063o			
158	R095o	D012o		
159	R093o			
160	R034o			DicDR8
161			Dic24o	DicR8
162	R049o	D079o	Dic15o	
163	R134r		Dic59r	
164	R130r			
165	R117r			
166	R123r			DicDR9
167		D036o		
168	R109o	D035o	Dic47o	
169	R118r			
170	R115o			
171		D026o		
172	R099o			
173	R082o			
174	R066o			
175		D027o		
176	R035o			
177	R027o			
178		D017o		
179	R116o	D028o	Dic6o	
180		D064o		
181		D053o		
182			Dic39o	
183	R080o			
184	R052o			DicR9
185	R029o	D030o		
186	R085o		Dic29o	
187	R097o			DR8
188	R031o			
189	R014o	D050o		DR9
190	R114o			
191	R041o	D083o		
192			Dic4o	
193	R181r			
194			Dic56r	
246	R144r			
247	R135r			
248	R022o			

Spot	*Rotenone*	*DNOC*	*Diclofenac*	*only EC_{10}*
198		D085o		
199		D0860		DicD1
200		D082o		
201		D062o	Dic51o	
202		D080o		
203		D088o		
204	R157r			
205	R003o			
206	R148r			
207	R030o			
208		D066o		
209		D072o		
210		D074o		
211		D037o		
212		D054o		
213	R101o			
214	R005o			
215	R156r			
216	R015o			
217		D070o		
218		D092o		
219		D045o		
220	R179r			
221	R198r			
222	R162r			DicR10
223	R166r			
224	R140r		Dic60r	
225	R167r	D094r		
226	R165r			
227			Dic34o	
228	R120r			
229	R151r		Dic53r	DicR11
230			Dic16o	
231	R072o		Dic17o	DicR12
232	R129r			DicR13
233	R173r		Dic22o	
234	R132r		Dic35o	DR10
235	R178r			
236	R145r	D106r		DR11
237	R177r			DR12
238	R013o	D078o		
239	R044o	D041o		
240			Dic20o	
241		D105r		
242			Dic50o	
243	R126r			
244	R124r			
245	R009o			
297	R187r			
298			Dic01o	
299	R036o			

Spot	*Rotenone*	*DNOC*	*Diclofenac*	*only EC_{10}*
249	R006o			
250	R125r			DR13
251	R205r			
252	R175r	D099r		
253		D097r		
254		D103r		
255		D110r		
256	R045o			
257		D023o		
258	R184r			
259	R007o			
260		D061o		
261		D025o		
262		D007o		
263		D001o		
264		D114r		
265	R038o			
266	R158r			
267	R103o			
268	R020o			
269		D098r		
270		D100r		
271			Dic21o	
272			Dic26o	
273		D061o		
274		D077o		
275	R138r			
276			Dic33o	
277		D065o		
278	R002o			
279		D095r		
280		D006o		
281		D020o		
282			Dic7o	
283	R073o			
284	R127r			
285	R152r			
286			Dic54r	
287		D087o		
288			Dic25o	
289		D073o		
290		D056o		
291		D075o		
292		D060o		
293	R033o			
294	R012o			
295			Dic45o	
296			Dic49o	
310		D025o		
311	R038r			
312		D104r		

Spot	*Rotenone*	*DNOC*	*Diclofenac*	*only EC_{10}*
300	R028o			
301	R053o			
302	R204r			
303	R061o			
304	R084o			
305	R108o			
306	R104o			
307	R058o			
308	R082o			
309	R174r			

Spot	*Rotenone*	*DNOC*	*Diclofenac*	*only EC_{10}*
313	R153r			
314	R092o			
315	R121r			
316		D109r		
317	R160r			
318		D033o		
319		D084o		
320		D029o		
321		D046o		
322		D015o		

II Results of Effect Screening Assays with Embryonal and Larval Stages of the Zebrafish (XVIIi)

Table F – Table K summarise percentages of observed effects in effect screening assays with embryos (2-50 hpf) or larvae (79-127 hpf) with three substances (Diclofenac, DNOC, Rotenone).

Abbreviations are:

BC:	no blood circulation
HB:	no heart beat
OE:	oedema
P:	pigmentation impairments
Ret:	Retardation in Development
C:	Coagulation
MF:	Malformations
H:	early hatching
HB <25/15s:	affected heartbeat (heart frequency below 100 heartbeats per minute)

Organisms with effects were counted and percentages of effects were calculated related to all exposed individuals. Up to three repetitions were performed (on different days) for each exposure condition (Test 1-3). Next to values for each test mean values (MV) from all tests are shown. Empty fields are adequate to 0 % observed effects.

Table F: Effects of 48 hour exposure of **Diclofenac** to larval stages of the zebrafish (79-127 hpf)

	Conc. [µM]	BC	HB	OE	P	Ret	C	HB <25/15s	MF
Test 1	**c**	5.56		5.56				5.56	
Test 2									
Test 3									
	MV	1.85	0.00	1.85	0.00	0.00	0.00	1.85	0.00
Test 1	**0.0014**								
Test 2									
Test 3									
	MV	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Test 1	**0.0029**	11.11		11.11				11.11	
Test 2									
Test 3									
	MV	3.70	0.00	3.70	0.00	0.00	0.00	3.70	0.00
Test 1	**0.0059**								
Test 2									
Test 3							11.11		
	MV	0.00	0.00	0.00	0.00	0.00	3.70	0.00	0.00
Test 1	**0.0115**	22.22		11.11				22.22	
Test 2									
Test 3				11.11					
	MV	7.41	0.00	7.41	0.00	0.00	0.00	7.41	0.00
Test 1	**0.0229**	44.44	33.33	44.44				11.11	44.44
Test 2		44.44	22.22	44.44			33.33	22.22	22.22
Test 3		11.11		11.11	11.11		22.22	11.11	
	MV	33.33	18.52	33.33	3.70	0.00	18.52	14.81	22.22
Test 1	**0.0459**	88.89	44.44	88.89			11.11	44.44	77.78
Test 2		55.56	33.33	55.56			44.44	22.22	55.56
Test 3		22.22	22.22	22.22			77.78		22.22
	MV	55.56	33.33	55.56	0.00	0.00	44.44	22.22	51.85
Test 1	**0.0918**	11.11	11.11	11.11			88.89		
Test 2		100.00	100.00	100.00					100.00
Test 3		66.67	66.67	66.67			33.33		66.67
	MV	59.26	59.26	59.26	0.00	0.00	40.74	0.00	55.56
Test 1	**0.1839**						100.00		
Test 2							100.00		
Test 3							100.00		
	MV	0.00	0.00	0.00	0.00	0.00	100.00	0.00	0.00

Table G: Effects of 48 hour exposure of **Diclofenac** to embryonal stages of the zebrafish (2-50 hpf)

	Conc. [µM]	*BC*	*HB*	*OE*	*P*	*Ret*	*C*	*HB <25/15s*	*MF*	*H*
Test1	**c**	5.56	5.56	5.56			5.56			
Test2										
Test3										
	MV	1.85	1.85	1.85	0.00	0.00	1.85	0.00	0.00	0.00
Test1	**0.0014**	11.11	11.11	11.11						
Test2							11.11			
Test3							11.11			
	MV	3.70	3.70	3.70	0.00	0.00	7.41	0.00	0.00	0.00
Test1	**0.0029**	33.33	22.22	11.11			11.11	11.11	11.11	
Test2							11.11			
Test3							11.11			
	MV	11.11	7.41	3.70	0.00	0.00	11.11	3.70	3.70	0.00
Test1	**0.0059**	11.11	11.11	11.11			11.11			
Test2										
Test3										
	MV	3.70	3.70	3.70	0.00	0.00	3.70	0.00	0.00	0.00
Test1	**0.0115**	77.78		88.89				11.11		
Test2		44.44		44.44			33.33	44.44		
Test3		55.56	11.11	55.56			22.22	44.44		
	MV	59.26	3.70	62.96	0.00	0.00	18.52	33.33	0.00	0.00
Test1	**0.0229**	100.00		100.00				100.00	44.44	
Test2		100.00		100.00				100.00	100.00	
Test3		100.00	55.56	100.00				44.44	100.00	
	MV	100.00	18.52	100.00	0.00	0.00	0.00	81.48	81.48	0.00
Test1	**0.0459**	100.00	66.67	100.00				33.33		
Test2		100.00	88.89	100.00				11.11	100.00	
Test3		100.00	77.78	100.00				22.22	100.00	
	MV	100.00	77.78	100.00	0.00	0.00	0.00	22.22	66.67	0.00
Test1	**0.0918**	100.00	100.00	100.00						
Test2		66.67	66.67	66.67			33.33		66.67	
Test3		77.78	77.78	77.78			22.22		77.78	
	MV	81.48	81.48	81.48	0.00	0.00	18.52	0.00	48.15	0.00
Test1	**0.1839**	66.67	66.67	66.67			33.33			
Test2		44.44	44.44	44.44			55.56		44.44	
Test3		55.56	55.56	55.56			44.44		55.56	
	MV	55.56	55.56	55.56	0.00	0.00	44.44	0.00	33.33	0.00

Table H: Effects of 48 hour exposure of **DNOC** to larval stages of the zebrafish (79-127 hpf)

	Conc [µM]	*BC*	*HB*	*OE*	*P*	*Ret*	*C*	*Hz <25/15s*	*MF*
Test1	**c**			2.77			5.56		
Test2									
Test3		-	-	-	-	-	-	-	-
	MV	0.00	0.00	1.39	0.00	0.00	2.78	0.00	0.00
Test1	**0.050**								
Test2									
Test3		-	-	-	-	-	-	-	-
	MV	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Test1	**0.252**	-	-	-	-	-	-	-	-
Test2									
Test3		-	-	-	-	-	-	-	-
	MV	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Test1	**0.505**						10.00		
Test2									
Test3		-	-	-	-	-	-	-	-
	MV	0.00	0.00	0.00	0.00	0.00	5.00	0.00	0.00
Test1	**1.262**	-	-	-	-	-	-	-	-
Test2									
Test3									
	MV	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Test1	**2.019**	-	-	-	-	-	-	-	-
Test2									
Test3									
	MV	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Test1	**2.523**						20.00		
Test2									
Test3									
	MV	0.00	0.00	0.00	0.00	0.00	6.67	0.00	0.00
Test1	**3.028**	-	-	-	-	-	-	-	-
Test2		-	-	-	-	-	-	-	-
Test3							10.00		
	MV	0.00	0.00	0.00	0.00	0.00	10.00	0.00	0.00
Test1	**3.533**	-	-	-	-	-	-	-	-
Test2		-	-	-	-	-	-	-	-
Test3									
	MV	0.00	0.00	0.00	0.00	0.00	10.00	0.00	0.00
Test1	**0.785**	-	-	-	-	-	-	-	-
Test2									
Test3		-	-	-	-	-	-	-	-
	MV	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

	Conc [µM]	BC	HB	OE	P	Ret	C	Hz <25/15s	MF
Test1	4.038	-	-	-	-	-	-	-	-
Test2		-	-	-	-	-	-	-	-
Test3							50.00		
	MV	0.00	0.00	0.00	0.00	0.00	50.00	0.00	0.00
Test1	4.290	-	-	-	-	-	-	-	-
Test2		-	-	-	-	-	-	-	-
Test3							80.00		
	MV	0.00	0.00	0.00	0.00	0.00	80.00	0.00	0.00
Test1	4.542	-	-	-	-	-	-	-	-
Test2		-	-	-	-	-	-	-	-
Test3							100.00		
	MV	0.00	0.00	0.00	0.00	0.00	10.00	0.00	0.00
Test1	4.795	-	-	-	-	-	-	-	-
Test2		-	-	-	-	-	-	-	-
Test3							80.00		
	MV	0.00	0.00	0.00	0.00	0.00	80.00	0.00	0.00
Test1	5.047						90.00		
Test2		10.00		10.00			90.00		
Test3		-	-	-	-	-	-	-	-
	MV	5.00	0.00	5.00	0.00	0.00	90.00	0.00	0.00
Test1	7.57						100.00		
Test2		-	-	-	-	-	-	-	-
Test3							100.00		
	MV	0.00	0.00	0.00	0.00	0.00	100.00	0.00	0.00
Test1	50.469						100.00		
Test2		-	-	-	-	-	-	-	-
Test3		-	-	-	-	-	-	-	-
	MV	0.00	0.00	0.00	0.00	0.00	100.00	0.00	0.00

Table I: Effects of 48 hour exposure of **DNOC** to embryonal stages of the zebrafish (79-127 hpf)

	Conc. [µM]	*BK*	*HZ*	*OE*	*P*	*Ret*	*C*	*HB <25/15s*	*MF*	*H*
Test1	**c**						8.30			
Test2							10.00			
Test3							10.00			
	MV	0.00	0.00	0.00	0.00	0.00	9.43	0.00	0.00	0.00
Test1	**0.25**						10.00			
Test2							10.00			
Test3		-	-	-	-	-	-	-	-	-
	MV	0.00	0.00	0.00	0.00	0.00	10.00	0.00	0.00	0.00
Test1	**0.5**						20.00			
Test2							10.00			
Test3										
	MV	0.00	0.00	0.00	0.00	0.00	10.00	0.00	0.00	0.00
Test1	**1.01**						10.00			
Test2		-	-	-	-	-	-	-	-	-
Test3		-	-	-	-	-	-	-	-	-
	MW	0.00	0.00	0.00	0.00	0.00	10.00	0.00	0.00	0.00
Test1	**1.51**	20.00	20.00							
Test2		10.00		20.00			10.00	10.00		
Test3		30.00		30.00				55.56		
	MV	20.00	6.67	16.67	0.00	0.00	3.33	21.85	0.00	0.00
Test1	**2.02**	10.00	10.00					10.00		
Test2		20.00	20.00	20.00			20.00	20.00		
Test3		-	-	-	-	-	-	-	-	-
	MV	15.00	15.00	10.00	0.00	0.00	10.00	15.00	0.00	0.00
Test1	**2.52**	50.00	50.00		30.00			10.00		
Test2		70.00	50.00	50.00	10.00		10.00	20.00		10.00
Test3		30.00		10.00			10.00	30.00		
	MV	50.00	33.33	20.00	13.33	0.00	6.67	20.00	0.00	3.33
Test1	**3.03**	70.00	70.00		40.00	30.00	20.00			
Test2		-	-	-	-	-	-	-	-	-
Test3		-	-	-	-	-	-	-	-	-
	MV	70.00	70.00	0.00	40.00	30.00	20.00	0.00	0.00	0.00
Test1	**3.53**	-	-	-	-	-	-	-	-	-
Test2		70.00	70.00	70.00	70.00	40.00	30.00			
Test3		90.00	50.00	70.00			10.00		10.00	
	MV	53.33	40.00	46.67	23.33	13.33	13.33	0.00	3.33	0.00
Test1	**4.04**	10.00	10.00		30.00	30.00	60.00			
Test2		-	-	-	-	-	-	-	-	-
Test3		-	-	-	-	-	-	-	-	-
	MV	10.00	10.00	0.00	30.00	30.00	60.00	0.00	0.00	0.00

	Conc. [µM]	BK	HZ	OE	P	Ret	C	HB <25/15s	MF	H
Test1	4.54	-	-	-	-	-	-	-	-	-
Test2		-	-	-	-	-	-	-	-	-
Test3						10.00	90.00			
	MV	0.00	0.00	0.00	0.00	10.00	90.00	0.00	0.00	0.00
Test1	5.05	-	-	-	-	-	-	-	-	-
Test2		-	-	-	-	-	-	-	-	-
Test3						0.00	100.00			
	MV	0.00	0.00	0.00	0.00	0.00	100.00	0.00	0.00	0.00
Test1	5.55	-	-	-	-	-	-	-	-	-
Test2		-	-	-	-	-	-	-	-	-
Test3						0.00	100.00			
	MV	0.00	0.00	0.00	0.00	0.00	100.00	0.00	0.00	0.00
Test1	15.14	-	-	-	-	-	-	-	-	-
Test2		-	-	-	-	-	-	-	-	-
Test3						0.00	100.00			
	MV	0.00	0.00	0.00	0.00	0.00	100.00	0.00	0.00	0.00
Test1	15.77	-	-	-	-	-	-	-	-	-
Test2		-	-	-	-	-	-	-	-	-
Test3						0.00	100.00			
	MV	0.00	0.00	0.00	0.00	0.00	100.00	0.00	0.00	0.00
Test1	31.54	-	-	-	-	-	-	-	-	-
Test2		-	-	-	-	-	-	-	-	-
Test3						0.00	100.00			
	MV	0.00	0.00	0.00	0.00	0.00	100.00	0.00	0.00	0.00
Test1	63.09	-	-	-	-	-	-	-	-	-
Test2		-	-	-	-	-	-	-	-	-
Test3						0.00	100.00			
	MV	0.00	0.00	0.00	0.00	0.00	100.00	0.00	0.00	0.00
Test1	126.17	-	-	-	-	-	-	-	-	-
Test2		-	-	-	-	-	-	-	-	-
Test3						0.00	100.00			
	MV	0.00	0.00	0.00	0.00	0.00	100.00	0.00	0.00	0.00
Test1	252.25	-	-	-	-	-	-	-	-	-
Test2		-	-	-	-	-	-	-	-	-
Test3						0.00	100.00			
	MV	0.00	0.00	0.00	0.00	0.00	100.00	0.00	0.00	0.00

Table J: Effects of 48 hour exposure of **Rotenone** to larval stages of the zebrafish (79-127 hpf)

	Conc. [nM]	BC	HB	OE	P	Ret	C	HB <25/15s	MF
Test1 Test2 Test3	c	4.10	4.10	4.10					
	MV	1.37	1.37	1.37	0.00	0.00	0.00	0.00	0.00
Test1 Test2 Test3	0.25	-	-	-	-	-	11.10 -	-	-
	MV	0.00	0.00	0.00	0.00	0.00	5.55	0.00	0.00
Test1 Test2 Test3	12.68						11.10		
	MV	0.00	0.00	0.00	0.00	0.00	3.70	0.00	0.00
Test1 Test2 Test3	25.35	-	-	-	-	-	11.10 -	-	-
	MV	0.00	0.00	0.00	0.00	0.00	3.70	0.00	0.00
Test1 Test2 Test3	38.03	- -	- -	- -	- -	- -	- -	- -	- -
	MV	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Test1 Test2 Test3	50.71	-	-	-	-	-	- 22.22 22.22	-	-
	MV	0.00	0.00	0.00	0.00	0.00	22.22	0.00	0.00
Test1 Test2 Test3	57.05	- -	- -	- -	- -	- -	- -	- -	- -
	MV	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Test1 Test2 Test3	63.39	- -	- -	- -	- -	- -	- - 66.67	- -	- -
	MV	0.00	0.00	0.00	0.00	0.00	66.67	0.00	0.00
Test1 Test2 Test3	69.73	- -	- -	- -	- -	- -	- - 55.56	- -	- -
	MV	0.00	0.00	0.00	0.00	0.00	55.56	0.00	0.00
Test1 Test2 Test3	76.06	-	-	-	-	-	- 100.00 88.89	-	-
	MV	0.00	0.00	0.00	0.00	0.00	94.44	0.00	0.00

	Conc. [nM]	BC	HB	OE	P	Ret	C	HB <25/15s	MF
Test1	101.42	-	-	-	-	-	-	-	-
Test2							100.00		
Test3		-	-	-	-	-	-	-	-
	MV	0.00	0.00	0.00	0.00	0.00	100.00	0.00	0.00
Test1	126.77						100.00		
Test2		-	-	-	-	-	-	-	-
Test3		-	-	-	-	-	-	-	-
	MV	0.00	0.00	0.00	0.00	0.00	100.00	0.00	0.00
Test1	253.55						100.00		
Test2		-	-	-	-	-	-	-	-
Test3		-	-	-	-	-	-	-	-
	MV	0.00	0.00	0.00	0.00	0.00	100.00	0.00	0.00
Test1	1267.75						100.00		
Test2		-	-	-	-	-	-	-	-
Test3		-	-	-	-	-	-	-	-
	MV	0.00	0.00	0.00	0.00	0.00	100.00	0.00	0.00
Test1	2535.50						100.00		
Test2		-	-	-	-	-	-	-	-
Test3		-	-	-	-	-	-	-	-
	MV	0.00	0.00	0.00	0.00	0.00	100.00	0.00	0.00

Table K Effects of 48 hour exposure of **Rotenone** to embryonal stages of the zebrafish (2-50 hpf)

	Conc. [nM]	*BC*	*HB*	*OE*	*P*	*Ret*	*C*	*HB <25/15s*	*MF*	*H*
Test1	**c**						10.00			
Test2		3.71	3.71	3.71			3.71			
Test3							3.71			
	MV	1.24	1.24	1.24	0.00	0.00	5.81	0.00	0.00	0.00
Test1	**0.0025**	10.00		10.00			10.00			10.00
Test2										
Test3							11.11			
	MV	3.33	0.00	3.33	0.00	0.00	7.04	0.00	0.00	3.33
Test1	**0.127**	-	-	-	-	-	-	-	-	-
Test2							11.11			
Test3										
	MV	0.00	0.00	0.00	0.00	0.00	3.70	0.00	0.00	0.00
Test1	**1.27**	10.00		10.00		10.00	10.00			
Test2		-	-	-	-	-	-	-	-	-
Test3		-	-	-	-	-	-	-	-	-
	MV	10.00	0.00	10.00	0.00	10.00	10.00	0.00	0.00	0.00
Test1	**2.54**			10.00	10.00	10.00	20.00			
Test2							11.11			
Test3							11.11			
	MV	0.00	0.00	3.33	3.33	3.33	14.07	0.00	0.00	0.00
Test1	**12.68**	10.00		10.00	60.00	50.00	20.00	10.00	20.00	
Test2		-	-	-	-	-	-	-	-	-
Test3		-	-	-	-	-	-	-	-	-
	MV	10.00	0.00	10.00	60.00	50.00	20.00	10.00	20.00	0.00
Test1	**50.71**						100.00			
Test2										
Test3							11.11			
	MV	0.00	0.00	0.00	0.00	0.00	37.04	0.00	0.00	0.00
Test1	**126.77**						100.00			
Test2										
Test3							11.11			
	MV	0.00	0.00	0.00	0.00	0.00	37.04	0.00	0.00	0.00
Test1	**190.16**						100.00			
Test2		11.11		11.11			11.11	11.11		
Test3										
	MV	3.70	0.00	3.70	0.00	0.00	37.04	3.70	0.00	0.00
Test1	**253.55**						100.00			
Test2		100.00	22.22	11.11	66.67	66.67	33.33	44.44		
Test3		44.44		11.11	33.33	33.33	11.11	33.33		
	MV	48.15	7.41	7.41	33.33	33.33	48.15	25.93	0.00	0.00

	Conc. [nM]	BC	HB	OE	P	Ret	C	HB <25/15s	MF	H
Test1	**507.1**	-	-	-	-	-	-	-	-	-
Test2		100.00	100.00		100.00	100.00				
Test3		88.89	88.89		88.89	88.89	11.11			
	MV	62.96	62.96	0.00	62.96	62.96	3.70	0.00	0.00	0.00

Publications

Gündel U, Benndorf D, von Bergen M, Altenburger R, Küster E. 2007. Vitellogenin cleavage products as indicators for toxic stress in zebrafish embryos: A proteomic approach. Proteomics 7: 4541-4554; DOI: 10.1002/pmic.200700381

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Gündel U, Benndorf D, Küster E. May 2006. Proteom analysis of Zebrafish (*Danio rerio*) embryos: Initial characterisation and the effects of environmental stressors. Poster Presentation SETAC Europe 16th Annual Meeting in Den Haag, The Netherlands

Gündel U, Benndorf D, von Bergen M, Altenburger R, Küster E. May 2007. Proteomics approach to determine early biochemical effects of ethanol in zebra ish (*Danio rerio*) embryos. SETAC Europe 17th Annual Meeting in Porto, Portugal

Gündel U, Benndorf D, von Bergen M, Altenburger R, Küster E. September 2007. Downstream of Genomics: Proteomics in Zebrafisch Embryonen. Platform Presentation SETAC –GLB in Leipzig, Germany

Gündel U, von Bergen M, Altenburger R, Küster E. May 2008. Changed yolk utilisation processes indicate toxic stress in zebrafish embryos: a proteomic study. Platform Presentation SETAC Europe 17th Annual Meeting in Warsawa, Poland

Erklärungen

Hiermit erkläre ich, Ulrike Gündel, dass ich bisher keine weiteren Promotionsversuche unternommen habe und nicht im Besitz eines Doktrogrades bin.

Leipzig, den

Ort, Datum Unterschrift

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Arbeit selbständig durchgeführt und keine anderen als die angegeben Hilfsmittel verwendet habe.

Leipzig, den

Ort, Datum Unterschrift

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Lebenslauf

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