博士論文（要約）

Studies on the effects of tetrodotoxin on gene expression in pufferfish *Takifugu rubripes*

（テトロドトキシンがトラフグの遺伝子発現に及ぼす影響に関する研究）

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論文題目 Studies on the effects of tetrodotoxin on gene expression in pufferfish Takifugu rubripes

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As some chapters of this thesis are anticipated to be published in a paper in a scholarly journal, they cannot be published online. The paper is scheduled to be published within 5 years (before 2019/3/31).

The Chapters in question are:

Chapter 2, Section 1: Gene expression in liver tissues of wild *T. rubripes*

Chapter 3: *T. rubripes* gene expression in liver – *ex-vivo*

Chapter 4: General Conclusion

It is not yet decided which scholarly journal these parts will be published in, but in all likelihood the publication will take place in Toxicon, the official journal of The International Society on Toxinology.

以上

Abstract

Tetrodotoxin (TTX) is a potent blocker of voltage-gated, fast sodium channels. TTX is the toxic principle of pufferfish toxin, also known as fugu toxin. TTX accumulates in most marine pufferfish in a tissue specific way. Previous studies, in which nontoxic cultured pufferfish reared with nontoxic diets became toxic upon ingesting the toxic ovary of wild pufferfish, inferred that pufferfish accumulates TTX by the food chain, which is the widely accepted hypothesis today. However, the exact mechanisms involved in the accumulation of tetrodotoxin in pufferfish, and the role the toxin plays in the pufferfish body, still remain unclear, although various studies tried to shed light on these. Among various possible approaches in which such details can be elucidated, one fundamental way is to obtain more insight about the genes related to the toxicity of pufferfish. Therefore, we set our goal of this study to learn more about biological functions and physiological roles and effects of TTX from a gene expression perspective, using the liver as a central organ of a multitude of biological processes and its vital role in dealing with xenobiotic substances.

To achieve this goal, we identified differentially expressed genes between toxic and nontoxic *T. rubripes* liver tissues in three different approaches that are each more controlled than the previous one and complement each other. Two of these approaches consist of *in-vivo* experiments, of which the first one was performed using tissues of wild specimens of toxic and nontoxic pufferfish, and the second one being an TTX injection experiment in cultured nontoxic pufferfish. The third approach is a well-controlled *ex-vivo* experiment using a liver tissue assay.

By identifying these differentially expressed genes we aim to better understand the role of proteins involved in the pharmacokinetics of TTX in the pufferfish body, such as metabolic enzymes, binding proteins and specific transporters. Furthermore, differences in physiological status of the two groups are further discussed in this dissertation.

Chapter 2 contains both *in-vivo* experiments. 18 wild *T. rubripes* specimens were killed on location and tissue samples of liver were taken and stored in RNA*later* for subsequent total RNA extraction. Remaining liver tissues were analyzed for TTX content using a HPLC system with post column derivatization and fluorescence detection. Separation of TTX from other compounds was achieved based on the principle of ion-pair chromatography using 5 mM heptanesulfonic acid (HSA) as an ion-pair reagent in a mobile phase consisting of 1% (v/v) acetonitrile and 10 mM ammonium formate at pH 5.0. Separated compounds were treated with hot NaOH to facilitate degradation of TTX to detectable fluorescent derivatives. Based on quantitative TTX measurement results and total RNA quality, 4 toxic and 4 nontoxic liver tissue RNA samples were used for subsequent cDNA microarray experiments. The average toxicity of liver samples was 27.8 ± 15.0 μg TTX/g (127 ± 68 MU/g).

Total RNA samples were prepared for microarray analysis by reverse transcription and subsequent labeling with Cy3-labeled CTPs to fluorescent cRNAs. These probes were hybridized to microarrays, which

were custom designed from the *T. rubripes* transcriptome database. Expression data of transcripts was digitalized and differential expression between toxic and nontoxic samples was analyzed using different computational approaches. Differential expression analysis of single genes was performed with fold-change analysis using Agilent Technologies' GeneSpring GX software. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database and the Gene Ontology (GO) functional annotation vocabulary were used to gain further insight into underlying biological meaning and functional processes that would otherwise be difficult to extract from the list of single genes. Fisher's Exact test was used on GO data to find significantly enriched annotation terms among the expression data. 4,867 probe sets were found to be up-regulated and 4,535 were down-regulated in toxic liver tissues. Of these, 678 transcripts had a fold-change difference in expression of 2 or larger. 248 transcripts (144 genes) were up-regulated and 430 transcripts (291 genes) down-regulated. Among the up-regulated genes were vitellogenin 6, phospholipase A2 precursor, apolipoproteins A-IV1 and Bb, cytochrome P450 3A4 and glutathione S-transferase mu 3. The list of highly down-regulated gene products contained diacylglycerol kinase eta, transmembrane proteins 165 and 102, peroxisome proliferator-activated receptor gamma (PPARγ) coactivator 1-beta and NADH dehydrogenase subunits 1, 2 and 5.

GO analysis allowed for functional classification of differentially expressed transcripts in the three categories Biological Process, Molecular Function and Cellular Compartment. "Ion binding", "hydrolase activity" and "oxidoreductase activity" were the highest represented terms in the Molecular Function category. Many functional terms that are localized to mitochondria were found to be significantly enriched, as well as immune system and lipid binding and transport related processes. Purine and thiamine metabolism related pathways were among the highest represented KEGG pathways. These results suggest that natural occurring long-term exposure to TTX changes pufferfish physiology and metabolism on a gene expression level by modifying immune system and lipid related processes.

The second *in-vivo* experiment was performed under more controlled conditions by injecting TTX into the caudal muscle of cultured specimens of *T. rubripes* and investigating differential gene expression after 5 days. The 10 fish used in this study were 18 months old and had a body weight of approximately 1 kg. TTX was dissolved in modified Hank's balanced salt solution buffer and 0.25 mg TTX/kg body weight was injected into 5 of the fish. On the fifth day after administration, liver samples were prepared as previously described for subsequent quantitative TTX determination and for microarray analysis, with the 5 samples from TTX-injected fish as a test group, and 5 samples from buffer-only injected fish as a control group. After 5 days, the TTX content in livers was 28 ± 13% of the original dose.

In microarray analysis, probe sets with a p-value of 0.05 or smaller and a fold-change difference in expression of 2.0 or higher were selected as differentially expressed transcripts. 490 transcripts (466 genes) passed this filter. Of these, 63 transcripts (40 genes) were up-regulated and 427 transcripts (426 genes) down-

regulated. The list of up-regulated genes contained chymotrypsin-like elastase family member 2a, transmembrane protein 168, Rho GTPase-activating protein 29, calcium channel alpha-1 subunit homolog and nicotinic acetylcholine receptor alpha 9d subunit. Among the down-regulated genes, nuclear receptor coactivator 2, transcription factor SOX-5, hepatocyte nuclear factor 3-gamma and plasma membrane calcium-transporting ATPase 3 were highly differentially expressed, among others. Functional classification according to GO terminology did not differ much from the one in liver samples of wild *T. rubripes*, with the difference that among Molecular Function terms after "ion binding", "signal transducer activity" and "transferase activity" were the most highly represented terms, and "oxidoreductase activity" was the least represented one, suggesting a higher prevalence of signal transduction related differentially expressed genes (DEGs). According to Fisher's Exact test, none of the differentially expressed probe sets are significantly enriched in a particular functional group. KEGG pathway analysis results are similar to those of genes in wild liver, with purine and thiamine metabolism related pathways being highly represented among the tested genes. Since thiamine is necessary for biosynthesis of neurotransmitters like acetylcholine and plays an important role in neural health, a difference in its metabolism between toxic and nontoxic pufferfish is likely a response to TTX neurotoxicity. "T cell receptor signaling pathway" related transcripts were also enriched and suggest an effect of TTX on expression of immune system related genes.

Quantitative real-time PCR was used to measure the expression of one of the highest differentially expressed transcripts (ENSTRUT00000045544) in toxic and nontoxic liver tissues. The measured fold-change difference in expression of 27.22 ± 4.18 showed good correlation with the 37.6-fold difference measured by microarray analysis, and further validates the microarray obtained results.

In Chapter 3, TTX accumulation and differential gene expression in *T. rubripes* liver slices of an *ex-vivo* tissue experiment are investigated. The liver of one female specimen of cultured *T. rubripes* was dissected, perforated with sterile perfusion buffer and then cut into 1 mm thick slices of 8 mm diameter, which were incubated at 20°C on a 24-well culture plate in Leibovitz's L-15 medium containing antibiotics. After 24h pre-incubation, TTX was added to a final medium concentration of 50 μM and 200 μM to some of the slices. After 1, 5, and 24 hours incubation, all liver discs of the respective experimental group were harvested and cut into 3 parts, one for cell viability analysis, one for quantitative TTX analysis, and one for total RNA extraction and gene expression analysis. Cell viability was confirmed with alamarBlue cell viability reagent and TTX levels were measured with the HPLC-FLD system as described previously. TTX quantification demonstrates that the liver tissue slices actively took up TTX from the medium with a linear correlation between uptake amount and medium concentration at a certain time. After 24 hours, liver slices accumulated TTX at a concentration that is more than 4 times higher than surrounding medium concentration.

Total RNA was extracted from liver tissues and mRNA was isolated. In order to prepare a whole transcriptome library for next-generation sequencing, mRNA was fragmented, ligated with an adapter and

subjected to reverse transcription. Barcodes were attached to thus obtained cDNAs, and 9 each of the barcoded libraries pooled and sequenced using the IonProton™ sequencer and Ion PI™ sequencing chip. Sequence data was subjected to quality control and mapped against the Ensembl release 76 (October 2014) of the *T. rubripes* reference genome. RNA-Seq analysis was performed and normalized samples investigated for differential gene expression using the "Exact Test" for two group comparisons, as implemented in CLC Genomics Workbench Ver. 7. Samples with an absolute fold-change difference of 1.5 or higher and a p-value smaller than 0.01 were used in functional analysis with GO and KEGG, which was performed as described. Lists of significant DEGs were chosen for single gene analysis if they passed an FDR-corrected cut-off filter of 0.05. The number of DEGs increased with time and TTX dose and ranged from 182 in 1h 50 μM samples to 391 in samples incubated with 200 μM TTX for 24h. 13 DEGs showed a TTX dose-dependent effect in gene expression, among them ATP synthase, glutaredoxin, cytochrome c oxidase subunit VIIc and mitochondrial tRNAs. GO analysis showed only little difference in the functional groups that are expressed in each experimental group, suggesting little time-dependent differences in the functional quality of gene expression in reaction to TTX exposure. Most of the GO-terms that were found to be significantly enriched among DEGs are related to mitochondria. KEGG analysis results are similar, with oxidative phosphorylation related pathways being highly represented by DEGs. A closer investigation in the single-genes list identifies many down-regulated DEGs involved in mitochondria function, such as ATPases, cytochrome c oxidase subunits VIIa and VIIc, ubiquinol-cytochrome c reductase complex III subunit IV and many others. It is likely that the observed effects on gene expression related to ATP production in mitochondria and of cytochrome oxidases is directly connected to effects of TTX acting on voltage-dependent Na^+ channels in hepatocytes, with possible implications for energy metabolism and other Ca^{2+} signaling dependent processes. These results also suggest that although *T. rubripes* is widely immune to the toxic effects of TTX, TTX is still able to modify various physiological processes in the pufferfish body.

Lastly, a separate virtual experiment was set up to analyze the expression of Pufferfish Saxitoxin and Tetrodotoxin Binding Protein (PSTBP) homologs Tr1 and Tr3. Although previous studies found Tr1 and Tr3 to be only constitutively expressed, and no toxin-triggered change in expression was observed, we demonstrate in our study the TTX dose- and time-dependent up-regulation of Tr3 and simultaneous down-regulation of Tr1, while expression in control group remains unchanged. This result suggests a direct or indirect effect of TTX on the expression of these genes, which is likely to affect TTX accumulation and distribution in the pufferfish body by facilitating uptake into tissue.

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Chapter 1: General Introduction

History of Tetrodotoxin

Tetrodotoxin (TTX) is indispensable in a variety of scientific disciplines and applications. A PubMed search (*http://www.ncbi.nlm.nih.gov/pubmed*) for "tetrodotoxin" results in more than 18,400 hits (as of December 2014). Most of these hits are from publications in the field of neuroscience, in which TTX takes the role of a major tool in the study of ion channels, neurotransmission and synapse formation.

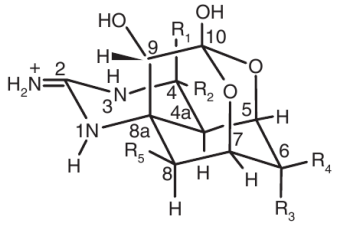
In Japan, the history of TTX goes back more than a century. Since the end of the 19th century there have been experiments on the isolation of the "puffer fish toxin", also known as "fugu poison"[1], and it was around that time that Dr. Tahara gave the toxin the name tetrodotoxin[2]. However, it took about another half century until it was first isolated and obtained in its crystallized form as the "poisonous principle of puffer toxin" by Yokoo in 1950[3]. 14 years later, three research groups succeeded in revealing the chemical structure and characteristics of TTX[1,4,5]. Another 8 years later, in 1972, the successful synthesis of racemic TTX was first reported[6].

Chemical properties

TTX is a low molecular mass substance with a molar mass of 319.27 g/mol and the molecular formula $C_{11}H_{17}O_8N_3$, whereby the free toxin exists in its hydratized form $C_{11}H_{17}O_8N_3 \cdot ½H_2O$ (M = 328.38 g/mol). The acid dissociation constant pK_a of TTX in water is 8.76 ± 0.01, and since it is insoluble in solvents which are not acids, it is clear that TTX is a weak base. Further studies have revealed that TTX is a zwitterionic compound with an acidic group and a basic guanidine group[1].

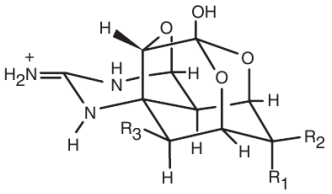
Besides from TTX itself, various of its analogs are contained in puffer toxin. The most prominent analogs are 4-*epi*tetrodotoxin and 4,9-anhydrotetrodotoxin, which have been first isolated and identified in 1985 and show about 6 times (710 MU/mg) and 49 times (92 MU/g) reduced toxicity compared to TTX, respectively[7]. The structures of hemilactal type TTX analogs including 4-*epi*TTX as well as the structures of 4,9-Anhydro type TTX analogs are shown in Figure 1[8].

(1) Hemilactal type



	R_1	R_2	R_3	R_4	R_5
TTX	H	OH	OH	CH_2OH	OH
4-*epi*TTX	OH	H	OH	CH_2OH	OH
6-*epi*TTX	H	OH	CH_2OH	OH	OH
11-*deoxy*TTX	H	OH	OH	CH_3	OH
6-*epi*-11-*deoxy*TTX	OH	H	OH	CH_3	OH
TTX-8-*O*-hemisuccinate	H	OH	OH	CH_2OH	$OOC(CH_2)_2COO^-$
				R *S*	
Chiriquitoxin	H	OH	OH	$CH(OH)CH(NH_3^+)COO^-$	OH
11-*nor*TTX-6(*S*)-ol	H	OH	OH	H	OH
11-*nor*TTX-6(*R*)-ol	H	OH	H	OH	OH
11-*nor*TTX-6,6-diol	H	OH	OH	OH	OH
11-*oxo*TTX	H	OH	OH	$CH(OH)_2$	OH
TTX-11-carboxylic acid	H	OH	OH	COO^-	OH

(2) 4,9-Anhydro type



	R_1	R_2	R_3
4,9-Anhydro TTX	OH	CH_2OH	OH
4,9-Anhydro-6-*epi*-TTX	CH_2OH	OH	OH
4,9-Anhydro-11-*deoxy*TTX	OH	CH_3	OH
4,9-Anhydro-TTX-8-*O*-*hemisuccinate*	OH	CH_2OH	$OOC(CH_2)_2COO^-$
4,9-Anhydro-TTX-11-*O*-*hemisuccinate*	OH	$CH_2OCO(CH_2)_2COO^-$	OH

Figure 1: Chemical structures of TTX and its analogs: (1) hemilactal type; (2) 4,9-Anhydro type[8]

Toxic potential and pharmacodynamics

Toxicological studies using a mouse bioassay standardized for TTX have been used to assess its level of toxicity, which is expressed as mouse units per mg TTX (MU/mg). Hereby, one mouse unit is the amount of toxin that kills a ddY strain mouse with a body weight of 19-20 g in 30 min after intraperitoneal injection

(i.p.). It has been found that TTX possesses a lethal potency of 4,500 MU/mg – in other words, 1 mg of TTX possesses the potency to kill 4,500 such mice in 30 min[7,9]. Therefore, simple mathematic operations allow us to define 1 MU TTX as 0.22 µg TTX. The medium lethal dose (LD_{50}) for a 50 kg male human is estimated to be 10,000 MU, which is equivalent to 2.2 mg TTX. This is a value which can be considered realistic based on experiences with previous deadly puffer poisoning incidents[10]. The material data safety sheet (MSDS) for TTX lists a LD_{50} for mice at 334 µg/g after oral application[11].

It was the discovery of the specific mechanism of TTX toxicity, namely the potent and selective blocking of fast, voltage-gated sodium channels (Na_V) that eventually led to a wide recognition of the possibilities of using TTX as a tool in physiological and pharmacological research[12,13]. TTX binds to the outer vestibule of sodium channels which contain the channel's selectivity filter for Na^+-ions, thereby blocking Na^+-flux and preventing the propagation of an action potential[14,15]. As a result, victims of TTX poisoning suffer from symptoms which are categorized into four degrees of gravity. Depending on dose, victims mainly suffer neuromuscular symptoms in rising intensity, starting from paresthesia of lips and tongue to paralysis of extremities and dysarthria and ataxia. Intoxication also exhibits symptoms of the cardiovascular system, leading to hypotension, hypertension or cardiac arrhythmias. Finally, death through TTX poisoning is usually caused by respiratory failure[16,17].

Furthermore, it is this specific blocking action of TTX that makes it valuable in the biochemical purification of Na_V channels, in the determination of Na_V channel density in cells or in the pharmacological characterization of sodium channels into TTX-resistant and TTX-sensitive channels[18].

So why is it, that pufferfish do not suffer from the same effects as, for example, mice or humans? In toxic pufferfish, parallel evolution of replacements at homologous pore-region residues in different gene paralogs of the voltage-gated sodium channels highly diminishes the affinity of TTX to bind to the channel[19], so that physiological function of fish remains healthy, even when high concentrations of TTX are present in their organs[20]. This resistance has been particularly well investigated with subunits NaV1.4 in skeletal muscle[21], but other channels were found to be resistant to TTX, as well, in varying degrees[19].

This resistance to TTX gives toxic pufferfish species almost immunity to TTX and it enables them to

accumulate the toxin in high concentrations, whereas nontoxic pufferfish as well as other nontoxic fish show no resistance and die[20,22]. However, even pufferfish that are known to be toxic in their natural habitat can die from TXX poisoning by artificially injecting high doses, the lethal dose being about 300-500 MU TTX/20 g body weight (i.p.) for *T. rubripes*[20].

Methods of detection and quantification

Considering the danger for human health and occasional deadly TTX poisoning incidents after ingestion of meals containing pufferfish parts, the importance of being able to detect and quantify TTX reliably has been understood and over the course of the years several biological and chemical qualitative and quantitative analysis methods have been developed.

Of those methods, some are used to identify TTX only qualitatively, among them being UV spectroscopy, IR spectrometry, gas chromatography – mass spectrometry (GC-MS), fast atom bombardment mass spectrometry (FABMS) and ^{1}H-NMR spectrometry[23]. Of those methods, ^{1}H-NMR played an especially important role in revealing the absolute configurations of the various derivatives of TTX that have been reported over time[7,8,24–31]. Both UV spectroscopy and GC-MS require a prior basic reaction of TTX with sodium hydroxide to form detectable quinazoline derivatives. This reaction is explained in more detail later.

Among the quantitative detection methods, the previously most commonly used detection method is the mouse bioassay. As has been mentioned in the introduction, to perform the mouse bioassay, a toxin extract is injected *i.p.* in ddY strain male mice. Toxicity can be quantified depending on the duration until time of death and is expressed in terms of mouse units (MU), whereby one mouse unit equals about 0.22 µg TTX[9]. However, due to reasons such as lack of accuracy, which stems from natural individual variances of the mice used, the inconvenience of keeping mice that fulfill the requirements of the assay, the nonspecifity of the assay to TTX and the constant endeavor to eliminate animal cruelty, focus has shifted to develop methods that surpass the mouse bioassay in accuracy as well as ease of use[32]. The most important among those detection methods are the cytotoxicity test, immunoassay, liquid chromatography – mass spectrometry (LC-MS) and high performance liquid chromatography (HPLC).

As cytotoxicity test, the tissue culture bioassay (TCBA) has been used. TCBA detects and quantifies

sodium channel blockers such as TTX based on their antagonizing ability on the combined effects of sodium channel activator veratridine and Na^+/K^+-ATPase inhibitor ouabain, which are applied to culture medium and cause the neuroblastoma cells used in this assay to round up an die. The antagonizing ability of TTX prolongs cell survival[33].

A variety of immunoassay methods for the detection of TTX have been developed and are still being improved. Frequently used is a competitive enzyme-linked immunoassay (EIA), which allows for rapid analysis in 30 minutes[34]. This method is based on an antibody developed from TTX-BSA conjugate injected Balb/c mice and has a detection range of 2-100 ng TTX/mL. More recently a antibody-based inhibition assay with a *surface plasmon resonance (SPR) sensor* has been developed and shows a detection range of 0.01 to 10,000 ng TTX/mL[35].

Two of the latest immunoassay methods to detect and quantify TTX are the gold nanoparticle probe-based immunoassay and the Fluidic Force Discrimination (FFD) assay. The gold nanoparticle probe-based immunoassay is a competitive immunoassay which uses gold nanoparticle-labeled monoclonal antibodies against TTX and is performed on a cellulose-nitrate membrane[36]. Major advantages of this assay are that it can be performed on location without need of any equipment, and that analysis takes only 10 minutes at a reasonably sensitive detection limit. Lastly, the FFD assay is a competitive immunoassay which uses a sandwich format with microbeads[37]. This assay shows an outstanding linear dynamic range over 4-5 orders of magnitude which would save time and sample, since it renders extensive dilution series to find the concentration range, in which TTX from samples can be measured confidently, unnecessary.

Finally, there are two analysis methods based on the principle of chromatography, one of them being HPLC coupled to a fluorescence detector (HPLC-FLD) and liquid chromatography coupled to a mass spectrometer (LC-MS). Next to the mouse bioassay, these two analysis methods are those most used so far and HPLC-FLD is method of choice in this study.

HPLC methods have been developed since 1982 and improved constantly[38]. All methods are generally based on the same principle: At first, the TTX in the sample is separated from other molecules in a buffer

system and on a stationary phase that can be silica gel, C-18 or an ion exchange resin[38–40]. Since TTX as it is cannot be detected by a fluorimeter, a post column reaction is introduced that triggers the reaction of TTX to a C_9-quinazoline derivative (2-amino-6-hydroxymethyl-8-hydroxyquinazoline), which can be excited to fluorescence at certain wave lengths (Figure 2)[1]. This reaction is usually performed with 4 N sodium hydroxide from a separate pump in a reaction oven at about 110°C and a reaction time of 2 minutes[41].

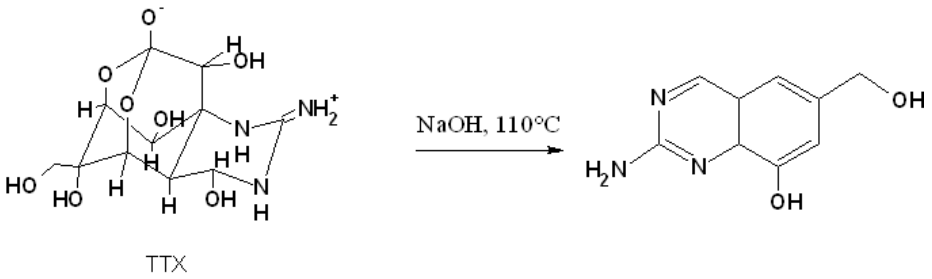


Figure 2: C9 quinazoline derivative of TTX, product of alkaline degradation[1]

Especially the reverse-phase ion-pair method enables separation of TTX from its analogs, in particular 6-*epi*TTX, 4-*epi*TTX and 4,9-anhydroTTX. As ion-pair reagent, heptanesulfonic acid (HSA) and ammonium heptafluorobutyrate have been successfully used[40,41].

One of the disadvantages of the HPLC-FLD system is the varying fluorescent intensity among TTX and its analogs. For example, the intensity of the alcalic degradation product of 11-deoxyTTX shows only 1/100th, but the product of 6-*epi*TTX 20-fold more intensity than the degradation product of TTX itself. This makes a simultaneous measurement of TTX and a specific analog in a sample difficult[41].

To bypass this problem and ensure that signals of different analogs are not mixed up in the analysis process, liquid chromatography methods have been combined with mass spectrometry. The so far most sophisticated mass spectrometry detection methods use electrospray ionization (ESI) for the ionization of TTX, whereby positive mode shows higher sensitivity than the negative mode[42]. As liquid chromatography system, these methods use either ion-pair chromatography on a reverse-phase resin or a cation-exchange separation column to decrease matrix effects which reduce the ionic strength of TTX. Detection is performed in either selected ion monitoring (SIM) mode for LC-ESI-MS or in selected reaction monitoring (SRM) mode in LC-ESI-

MS/MS mode. Since the ratio of peak area and applied amount of each analog shows less than 10% difference from the ratio of TTX, analogs can be quantified using the same calibration curve that is being used for TTX, which is the single biggest advantage over the LC-FLD method[41,42].

Accumulation in pufferfish and pharmacokinetics

The biological advantage of TTX accumulation is not fully understood so far, but it is mainly assumed that TTX plays a defensive and/or communicative function in pufferfish[43]. The antipredator function is assumed to work by TTX secretion from skin glands or by deterrence from eating toxic eggs. The communicative role of TTX might be one as a sex pheromone in some species.

Pufferfish have been shown to accumulate toxin in an exogenous manner instead of producing it themselves, likely through the food chain[44,45]. Various factors have led to this conclusion:

First of all, it has been found that pufferfish is not the only organism in which TTX is found. On the contrary, there is a multitude of different organisms that have high concentrations of TTX in their bodies, such as flatworms, ribbon worms, starfish, goby, xanthidae crabs and various amphibians such as frogs, newts, salamanders and snakes[46,47]. The fact that many different species and animals – marine and terrestrial – show high concentrations in TTX argue against the hypothesis, that TTX is produced by those species themselves by a common mechanism[48]. Furthermore, it has been shown that toxic pufferfish reared in net cages or on land under conditions, in which the invasion of TTX-bearing organisms is prevented, is nontoxic[49,50]. These pufferfish can be toxified again upon feeding with TTX-containing diet[51,52]. And lastly, marine bacteria that produce TTX were found in intestines of TTX-bearing organisms, although research showed that some of those produce TTX in limited quantity [53,54]. Also, there is at least one report that found TTX in cultured pufferfish *T. rubripes* using indirect competitive enzyme immunoassay (EIA) with a monoclonal antibody against TTX, even though the fish were hatched and cultured in a flow-through tank, under conditions that exclude the possibility of contact with TTX from exogenous sources. However, the measured amounts of TTX were even at the highest level in skin only about 0.2 MU/g tissue and therefore can be considered as being of minor importance[55].

TTX accumulates in pufferfish in a tissue specific manner, whereby marine pufferfish show a tendency to

accumulate the toxin in ovary and liver, whereas brackish water pufferfish tend to accumulate TTX in higher concentrations in skin[45]. But even within the group of marine pufferfish – to which the subject of this research, pufferfish *Takifugu rubripes*, also known as tiger puffer, belongs – different species show slightly different accumulation spectra, differing mainly in skin and muscle toxicity. Table 1 shows these spectra for a selection of marine pufferfish[45].

Table 1: Toxicity of selected marine pufferfish species (*Mar. Drugs* 2008, ***6***, 220–242); ×: <2 µg TTX/g (not toxic); ○: 2-19 µg TTX/g (weakly toxic); ❍: 20-199 µg TTX/g (moderately toxic); ●: >200 µg TTX/g (strongly toxic); —: data not available

Species	Maximal toxicity						
	Ovary	Testis	Liver	Skin	Intestine	Muscle	Blood
Takifugu rubripes	❍	×	❍	×	○	×	×
T. niphobles	●	○	●	❍	●	○	—
T. poecilonotus	●	❍	●	❍	❍	○	—
T. chrysops	❍	×	❍	❍	○	×	×
T. vermicularis	❍	×	❍	❍	○	×	—

Whereas the precise reasons behind this tissue specific accumulation pattern still remain unknown, various experiments have been undertaken to investigate the details of where exactly TTX accumulates and how it distributes among tissues short-term and long-term in the body of various pufferfish species.

In TTX application experiments, in which TTX has been applied into the digestive tract of nontoxic, cultured pufferfish *T. rubripes*, it has been shown that after uptake into blood circulation, TTX accumulates into liver in a first step[56,57]. This TTX uptake is presumably achieved by an active, carrier-mediated transport mechanism[58] which has been shown to accumulate TTX preferentially to structurally similar saxitoxins (STXs)[59].

In blood plasma, TTX has been shown to bind to specific binding proteins in *T. niphobles*[60] and *T. pardalis*[61]. In *T. rubripes*, the fraction of TTX not bound to any plasma proteins comprised 64% at a total TTX plasma concentration of 0.80 µg/mL[60].

In ovary of *T. vermicularis*, TTX accumulates at nucleus and yolk vesicles and granules[62]. In *T. niphobles* accumulation takes place at yolk globules and cytoplasm of preovulated oocytes and in the vitelline envelope

of ovulated oocytes, from where TTX is likely released during ovulation and functions as a pheromonal attractant to *T. niphobles* males[63].

Besides from the elimination of TTX from the oocytes in the described manner, elimination by secretion from specialized glands in the skin has been reported for various pufferfish species, but not for pufferfish *T. rubripes*, which does not possess such glands[64] and accumulates TTX after application of high doses only in juvenile fish presumably due to immature formation of skin tissue[65].

Finally, although information about metabolism of TTX in the pufferfish body is sparse, one possible metabolic pathway of TTXs has been shown to be via conjugation with cysteine or glutathione (GSH) at C4 to 4-CysTTX or 4-GSTTX as a first step. 4-GSTTX can be hydrolyzed by peptidases to 4-CysTTX in a secondary reaction[66].

Goals of this study

Looking at this current knowledge about TTX pharmacokinetics in *T. rubripes*, it becomes clear, that whereas the origin of TTX in pufferfish *T. rubripes* seems to be elucidated and many phenomena have been observed, there is no knowledge about specific mechanisms or molecules that explain the transfer, accumulation, metabolism and elimination mechanisms of TTX in the pufferfish body, aside from a possible role of a yet not fully classified fibrinogen-like protein[67] and data about the "pufferfish STX and TTX binding protein" (PSTBP)[61]. Also, it is yet unknown how exactly TTX as a xenobiotic in the pufferfish body influences other physiological factors and which role these play.

Therefore, we set our goals of this study to gain more insight into these mechanisms from a gene expression perspective, using the liver as a central organ of a multitude of biological processes and its vital role in dealing with xenobiotic substances. To achieve this goal, we identified differentially expressed genes between toxic and nontoxic *T. rubripes* liver tissues in three different approaches that are each more controlled than the previous one and complement each other. Two of these approaches consist of *in-vivo* experiments, of which the first one was performed using tissues of wild specimens of toxic and nontoxic pufferfish, and the second one being an TTX injection experiment in cultured nontoxic pufferfish. The third approach is a well-controlled *ex-vivo* experiment using a liver tissue assay.

By identifying these differentially expressed genes we aim to better understand the role of proteins involved in the pharmacokinetics of TTX in the pufferfish body, such as metabolic enzymes, binding proteins and specific transporters. Furthermore, differences in physiological status of the two groups are further discussed in this dissertation.

Chapter 2: *T. rubripes* gene expression in liver – *in vivo*

Section 2: Gene expression in liver tissues of cultured T. rubripes after TTX injection into caudal muscle

Introduction

Injection experiments of TTX into pufferfish have been performed before, e.g. in the determination of the toxicity threshold of the toxin to the almost immune fish, or in determination of important pharmacokinetics parameters. It is logical to use this method then to also determine the effects of TTX on gene expression *in-vivo* in a more controlled setting than in Section 1 with wild pufferfish.

Materials and Methods

Materials

T. rubripes specimens were cultured at the Aquaculture Station, Kawaku Co, Ltd. in Shimonoseki, Yamaguchi Prefecture, Japan by a flow-through aquaculture system which utilizes underground seawater from the Kanmon Tunnel (refer back to **Error! Reference source not found.**). The water temperature was kept constantly at around 20°C throughout the year and fish were fed with commercial diets. At sampling time in September 2010, the specimens of *T. rubripes* were 18 months old and all had a body weight of approximately 1 kg.

TTX used for injection was purified from ovaries of wild pufferfish *T. rubripes* collected in Genkai-nada Sea (**Error! Reference source not found.**) by ultrafiltration through a YM-1 Ultracel regenerated cellulose filter with 1 kDa molecular weight limit (Milipore, Bedford, MA, USA) and consecutive series of column chromatography steps on a Bio-Gel P-2 column (Bio-Rad Laboratories, Hercules, CA, USA) and a Bio-Rex 70 column (Bio-Rad Laboratories), as reported Nagashima et al.[72].

TTX used as standard for HPLC-FLD analysis was obtained commercially (Wako Pure Chemicals Industries, Osaka, Japan). All other reagents were of analytical grade quality.

TTX administration and sampling

TTX was dissolved in modified Hank's balanced salt solution buffer (supplementary information) and

applied to the 5 specimens (mean body weight 0.99 ± 0.06 kg) by intramuscular injection of 500 µL buffer/kg body weight, which contained an equivalent of 0.25 mg TTX/kg body weight, into the caudal muscle. 5 different specimens (mean body weight 1.02 ± 0.06 kg) were designated as control group and injected with 500 µL buffer/kg body weight without TTX. The fish were maintained in a 1,000 L circular culture tank for 5 days at 20°C, using the previously described flow-through aquaculture system. On the fifth day after administration, fish were removed from the culture tank and dissected. Total organs were weighed and liver tissue samples for subsequent RNA experiments taken and stored in RNAlater solution as described in Section 1: Materials and methods. Tissue samples destined for quantitative TTX analysis were stored at -20°C.

Quantitative TTX analysis

TTX was extracted from liver tissues as described in Section 1: Materials and methods.

Preparation of Cy3 labeled cRNA and microarray analysis

Extraction of total RNA, quality control and the preparation of Cy3 labeled cRNA was performed as described in Section 1: Materials and methods.

Data analysis was basically performed as described in Section 1, with selection of differentially expressed probe sets based on a fold-change difference cut-off value set at 2.0. Differing from the previously described analysis parameters, not all probe sets with less than 75% present or marginal features were discarded. Instead, probe sets with at least one feature present or marginal in both toxic and control groups were considered. Additionally, only probe sets with a p-value of 0.05 or smaller, as calculated by an unpaired Student's t-test in GeneSpring GX software version 11.0, were selected for differential gene expression experiments.

Quantitative real-time PCR

Quantitative real-time PCR was used to further validate the differential expression of transcript ENSTRUT00000045544 (chymotrypsin-like elastase family member 2A), which was found to be one of the highest up-regulated transcripts in the TTX-administered group by the microarray analysis. Total RNA was

extracted as described above and treated with DNase I (Invitrogen, Carlsbad, CA, USA). First-strand cDNAs were constructed using oligo-dT20 primers and SuperscriptTM III reverse transcriptase (Invitrogen) according to the manufacturer's protocol. Real-time PCR was performed in a 20-μL reaction mixture containing a 1:20 dilution of cDNA, 10 μL SYBR Premix Ex Taq II polymerase mix (TaKaRa Bio, Otsu, Japan), 0.4 μL ROX Reference Dye (TaKaRa Bio), 0.8 μL of gene-specific forward primer and 0.8 μL gene-specific reverse primer, both at a concentration of 10 μM. The reaction was performed on an ABI7300 Real-Time PCR System (Applied Biosystems) set to 95°C for 30 s, then 40 cycles of 95°C for 5 s and 60°C for 31 s. Relative-fold change difference of target mRNA expression was calculated with the comparative delta threshold cycle (ΔCt) method for relative quantification, with *T. rubripes* beta actin 1 mRNA (accession number U37499.1) expression as internal control. The gene-specific primers were designed using Primer Express software version 3.0 (Applied Biosystems).

Results

Sampling and quantitative TTX analysis

Body weights of the specimens of the TTX administration group and corresponding calculated doses of injected TTX are summarized in Table 2. The mean accumulation of TTX in livers on the fifth day after injection was 66 ± 12 ug TTX, which is 28 ± 13% of the original dose.

No TTX could be detected in the control group.

Table 2: Quantitative TTX measurement results in liver tissues of the TTX administration group

sample #	body weight [g]	dose (μg)	TTX conc. [ug/g]	TTX amount (μg)	accumulation (%)
1	1140	285	0.48	49	17.2
2	840	210	0.68	42	20.0
3	1000	250	1.12	93	37.2
4	1100	275	0.54	51	18.5
5	860	215	1.35	96	44.7

Microarray analysis

Preparation of Cy3 labeled cRNA

Spectrophotometric analysis of all total RNA samples showed a minimum A260/280 ratio of 2.0 and larger. rRNA bands of 28S and 18S rRNA could be confirmed in the denaturing agarose gel electrophoresis. cRNA yield and specific Cy3 activity of each sample is summarized in Table 3.**Error! Reference source not found.**

Table 3: cRNA yield and specific Cy3 activity of samples used for microarray analysis; *FOI* – frequency of incorporation, *L1-L5* – TTX administered group, *cL1-cL4* – control group

sample	FOI (Cy3)	cRNA yield [µg]	specific activity [pmol/µg]
L1	3.508	8.3	10.8
L2	3.290	12.8	10.1
L3	3.337	21.4	10.3
L4	2.671	12.4	8.1
L5	3.029	15.0	9.3
cL1	3.221	13.1	9.9
cL2	2.861	11.4	8.8
cL3	3.038	13.9	9.4
cL4	3.241	15.0	10.0
cL5	3.156	15.4	9.7

Gene Ontology analysis

Gene ontology results are summarized in Figure 3. The highest represented GOSlim terms are "cellular process", "single-organism process" and "metabolic process" in the Biological Process (level 2) category. In the Molecular Function annotation category on level 3, "ion binding" had the most annotated results, followed by an equal contribution of the terms "signal transducer activity", "transferase activity", "heterocyclic compound binding", "organic cyclic compound binding", "hydrolase activity" and "transmembrane transporter activity." The annotations least represented were "protein binding" and "oxidoreductase activity."

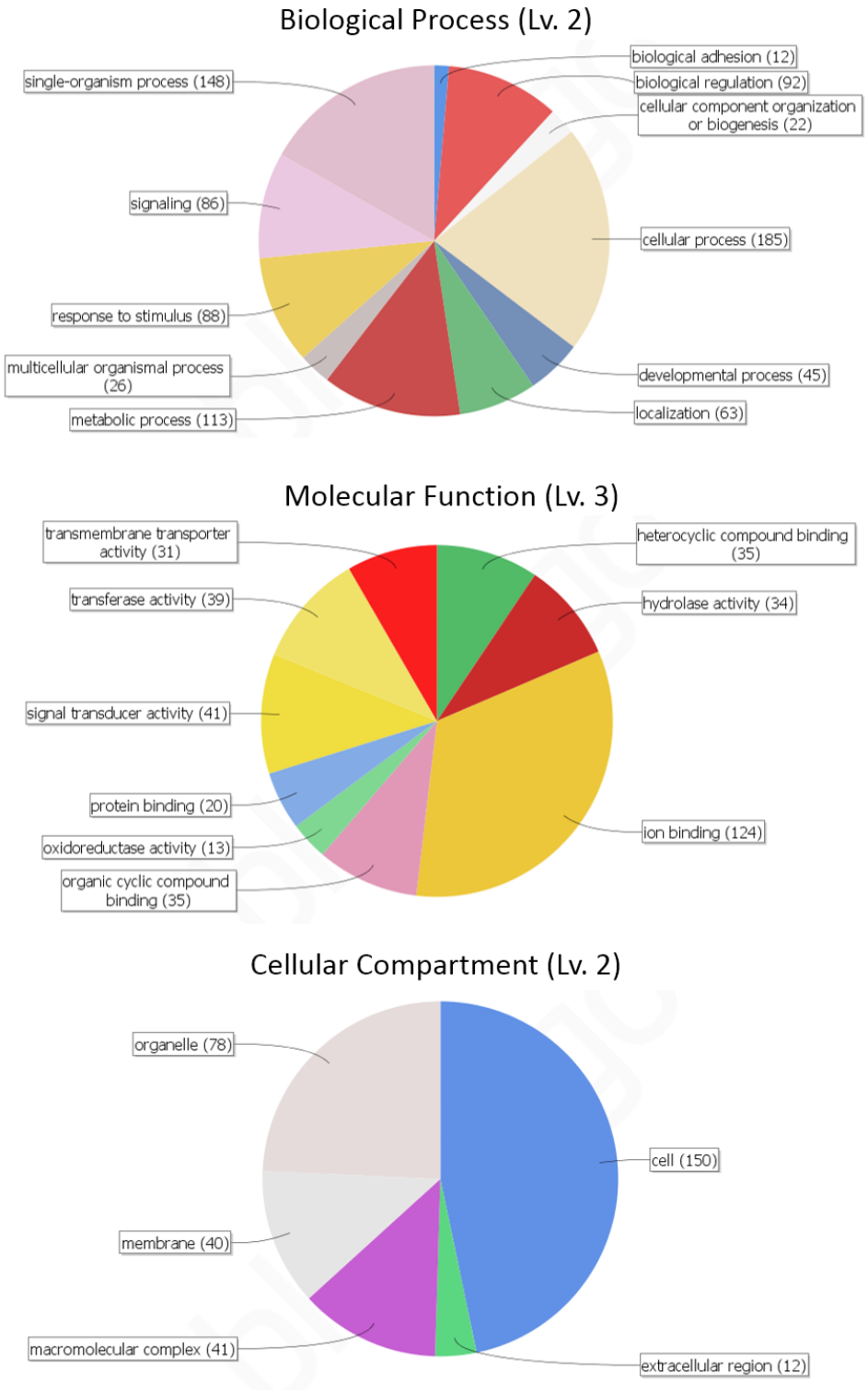


Figure 3: Gene Ontology (GO) for DEGs after TTX injection in cultured *T. rubripes*; number of mapped transcripts is shown in brackets

According to Fisher's Exact test, none of the DEGs are significantly enriched at a FDR cut-off at 0.05. The same test was repeated at a less stringent cut-off value of 0.1, but again no significant enrichment was found.

KEGG pathway analysis

KEGG pathway analysis revealed the highest functional representation of the pathways "purine metabolism" (19 transcripts) and "thiamine metabolism" (16 transcripts) among the differentially expressed genes. Five transcripts belonged to the "T cell receptor signalling pathway" and three transcripts to the "phosphatidylinositol signaling system", while all other pathway hits were represented by two or one transcripts.

Table 4: KEGG pathway analysis results for differentially expressed transcripts after TTX administration in cultured pufferfish *T. rubripes*

Pathway	**transcripts**
Purine metabolism	19
Thiamine metabolism	16
T cell receptor signaling pathway	5
Phosphatidylinositol signaling system	3
One carbon pool by folate	2
Glycerophospholipid metabolism	2
Glycerolipid metabolism	2
Steroid hormone biosynthesis	2
Pyrimidine metabolism	2
D-Arginine and D-ornithine metabolism	1
Carbon fixation pathways in prokaryotes	1
Arginine and proline metabolism	1
Inositol phosphate metabolism	1
mTOR signaling pathway	1
Betalain biosynthesis	1
Penicillin and cephalosporin biosynthesis	1
Lysine degradation	1
Glycine, serine and threonine metabolism	1
Glycosphingolipid biosynthesis - ganglio series	1
Nitrogen metabolism	1
Tyrosine metabolism	1

Fold-change analysis

490 transcripts passed the fold-change and p-value filters and were defined as differentially expressed. Of

these transcripts, 63 were up-regulated and 427 down-regulated. When investigating how many genes are involved in the transcript expression, we find these transcripts are encoded by 466 unique genes, of which 40 were up-regulated and 426 down-regulated (Table 5 and Table 6).

Table 5: Top 40 up-regulated transcripts by fold-change (fc) in TTX administration group

fc	Description	Ensembl Transcript ID	Ensembl Gene ID
70.92	chymotrypsin-like elastase family member 2a-like	ENSTRUT00000045548	ENSTRUG00000017710
49.98	chymotrypsin-like elastase family member 2a-like	ENSTRUT00000045547	ENSTRUG00000017710
48.11	chymotrypsin-like elastase family member 2a-like	ENSTRUT00000045546	ENSTRUG00000017710
43.13	chymotrypsin-like elastase family member 2a-like	ENSTRUT00000045545	ENSTRUG00000017710
37.56	chymotrypsin-like elastase family member 2a-like	ENSTRUT00000045544	ENSTRUG00000017710
19.98	Transmembrane protein 168	ENSTRUT00000041722	ENSTRUG00000016264
12.11	Rho GTPase-activating protein 29	ENSTRUT00000006023	ENSTRUG00000002577
10.35	Calcium channel alpha-1 subunit homolog (Fragment).	ENSTRUT00000034782	ENSTRUG00000013571
8.08	Nicotinic acetylcholine receptor alpha 9d subunit (Fragment).	ENSTRUT00000004724	ENSTRUG00000002046
6.97	CUB and sushi domain-containing protein 2	ENSTRUT00000038610	ENSTRUG00000015057
6.79	Serine incorporator 4	ENSTRUT00000036792	ENSTRUG00000014342
6.64	Pecanex-like protein C14orf135 (Hepatitis C virus F protein-binding protein 2)	ENSTRUT00000026094	ENSTRUG00000010325
5.44	Low-density lipoprotein receptor-related protein 2 Precursor (Megalin)	ENSTRUT00000046072	ENSTRUG00000017911
5.21	Collagen alpha-1(XXVII) chain Precursor	ENSTRUT00000046718	ENSTRUG00000018194
4.50	Kelch-like protein 32 (BTB and kelch domain-containing protein 5)	ENSTRUT00000000397	ENSTRUG00000000186
4.31	ELK4, ETS-domain protein	ENSTRUT00000000173	ENSTRUG00000000077
4.29	Zinc finger and BTB domain-containing protein 45 (Zinc finger protein 499)	ENSTRUT00000025902	ENSTRUG00000010240
4.28	SPRY domain-containing SOCS box protein 1 (SSB-1)	ENSTRUT00000020252	ENSTRUG00000008088
4.14	Dysferlin (Dystrophy-associated fer-1-like protein)(Fer-1-like protein 1)	ENSTRUT00000032342	ENSTRUG00000012731
4.03	Sodium channel subunit beta-2 Precursor	ENSTRUT00000003310	ENSTRUG00000001428
3.92	Lysyl oxidase homolog 2 Precursor	ENSTRUT00000011167	ENSTRUG00000004680
3.88	diencephalon/mesencephalon homeobox 1	ENSTRUT00000017057	ENSTRUG00000006916
3.86	Dual specificity protein kinase CLK2	ENSTRUT00000023088	ENSTRUG00000009138
3.77	Myosin-IIIB (EC 2.7.11.1)	ENSTRUT00000045827	ENSTRUG00000017818
3.68	Multiple epidermal growth factor-like domains 11 Precursor	ENSTRUT00000025143	ENSTRUG00000009956
3.66	Choline transporter-like protein 1 (Solute carrier family 44 member 1)	ENSTRUT00000003539	ENSTRUG00000001528
3.53	carbonic anhydrase IV a	ENSTRUT00000017798	ENSTRUG00000007204
3.50	odorant receptor, family F, subfamily 115, member 15	ENSTRUT00000006962	ENSTRUG00000002964
3.40	Membrane-associated phosphatidylinositol transfer protein 2	ENSTRUT00000024316	ENSTRUG00000009641
3.39	Platelet-activating factor receptor (PAF-R)	ENSTRUT00000030952	ENSTRUG00000012180
3.37	HEPACAM family member 2 Precursor	ENSTRUT00000038903	ENSTRUG00000015175
3.33	Probable G-protein coupled receptor 22	ENSTRUT00000043658	ENSTRUG00000016992
3.26	Tyrosine-protein phosphatase non-receptor type 2	ENSTRUT00000031306	ENSTRUG00000012316
3.24	Epidermal growth factor receptor substrate 15-like 1	ENSTRUT00000019629	ENSTRUG00000007862
3.19	zinc finger, DHHC-type containing 8	ENSTRUT00000036590	ENSTRUG00000014261
3.19	GTPase RhebL1 Precursor (Ras homolog enriched in brain-like protein 1)	ENSTRUT00000031045	ENSTRUG00000012211
3.03	Arf-GAP, GTPase, ANK repeat and PH domain-containing protein 2 (AGAP-2)	ENSTRUT00000047583	ENSTRUG00000018522
3.03	Peroxisome proliferator activated receptor gamma coactivator 1 alpha (Fragment)	ENSTRUT00000017824	ENSTRUG00000007210
3.02	6-phosphofructokinase type C	ENSTRUT00000027961	ENSTRUG00000011032
3.02	Transcription factor E2F2	ENSTRUT00000029743	ENSTRUG00000011729

fc	Description	Ensembl Transcript ID	Ensembl Gene ID
-12.95	Elongation factor G 2, mitochondrial Precursor (mEF-G 2)	ENSTRUT00000047556	ENSTRUG00000018513
-7.82	R-spondin-3 Precursor	ENSTRUT00000009260	ENSTRUG00000003904
-7.27	Nuclear receptor coactivator 2 (NCoA-2)	ENSTRUT00000029878	ENSTRUG00000011789
-6.23	liver carboxylesterase 2-like	ENSTRUT00000005082	ENSTRUG00000002200
-5.94	g protein-activated inward rectifier potassium channel 1-like	ENSTRUT00000042623	ENSTRUG00000016607
-5.73	Polypeptide N-acetylgalactosaminyltransferase 1	ENSTRUT00000029012	ENSTRUG00000011442
-5.49	Serine/threonine kinase 11-interacting protein (LKB1-interacting protein 1)	ENSTRUT00000034755	ENSTRUG00000013564
-5.34	Netrin receptor UNC5D Precursor (Protein unc-5 homolog D)	ENSTRUT00000007314	ENSTRUG00000003106
-4.74	DNA (cytosine-5)-methyltransferase 3A (Dnmt3a)	ENSTRUT00000024559	ENSTRUG00000009738
-4.72	tripartite motif containing 47	ENSTRUT00000028271	ENSTRUG00000011159
-4.42	Synaptopodin-2 (Myopodin)	ENSTRUT00000021561	ENSTRUG00000008568
-4.37	Kelch-like protein 8	ENSTRUT00000035155	ENSTRUG00000013714
-4.30	Ligand of Numb protein X 2 (Numb-binding protein 2)	ENSTRUT00000013283	ENSTRUG00000005500
-4.07	Transcription factor SOX-5	ENSTRUT00000005282	ENSTRUG00000002282
-4.02	Early growth response protein 3 (EGR-3)	ENSTRUT00000004950	ENSTRUG00000002142
-4.01	Nucleoside diphosphate kinase A (NDP kinase A)	ENSTRUT00000001876	ENSTRUG00000000779
-3.88	Cell adhesion molecule 2 Precursor (Immunoglobulin superfamily member 4D)	ENSTRUT00000037748	ENSTRUG00000014715
-3.83	Battenin (Protein CLN3)(Batten disease protein)	ENSTRUT00000029148	ENSTRUG00000011498
-3.75	Probable E3 ubiquitin-protein ligase HECTD3	ENSTRUT00000018412	ENSTRUG00000007419
-3.73	WD repeat domain phosphoinositide-interacting protein 2 (WIPI-2)	ENSTRUT00000010231	ENSTRUG00000004292
-3.69	Angiopoietin-2 Precursor (ANG-2)	ENSTRUT00000038056	ENSTRUG00000014844
-3.61	f-type lectin	ENSTRUT00000011781	ENSTRUG00000004908
-3.56	Tyrosine-protein kinase receptor TYRO3 Precursor	ENSTRUT00000025271	ENSTRUG00000010003
-3.44	Procollagen C-endopeptidase enhancer 1 Precursor	ENSTRUT00000036834	ENSTRUG00000014354
-3.40	Regulating synaptic membrane exocytosis protein 1 (Rab3-interacting molecule 1)	ENSTRUT00000009581	ENSTRUG00000004020
-3.36	PDZ and LIM domain protein 5 (Enigma homolog)	ENSTRUT00000013940	ENSTRUG00000005717
-3.34	Hepatocyte nuclear factor 3-gamma (HNF-3-gamma)	ENSTRUT00000041778	ENSTRUG00000016283
-3.33	Protein FAM70A	ENSTRUT00000026385	ENSTRUG00000010438
-3.24	Rho guanine nucleotide exchange factor (GEF) 26	ENSTRUT00000003229	ENSTRUG00000001394
-3.21	Kinesin-like protein KIF2C	ENSTRUT00000007982	ENSTRUG00000003387
-3.20	Ligand of Numb protein X 2	ENSTRUT00000013282	ENSTRUG00000005500
-3.19	Beta-crystallin A2	ENSTRUT00000005850	ENSTRUG00000002508
-3.13	Ectodysplasin-A receptor-associated adapter protein	ENSTRUT00000003860	ENSTRUG00000001675
-3.12	Pre-B-cell leukemia transcription factor-interacting protein 1	ENSTRUT00000015819	ENSTRUG00000006435
-3.11	serpin h1-like	ENSTRUT00000011222	ENSTRUG00000004704
-3.09	Plasma membrane calcium-transporting ATPase 3 (PMCA3)	ENSTRUT00000020096	ENSTRUG00000008036
-3.08	Plexin domain-containing protein 1 Precursor (Tumor endothelial marker 7)	ENSTRUT00000009874	ENSTRUG00000004152
-3.07	Sulfite oxidase, mitochondrial Precursor	ENSTRUT00000047512	ENSTRUG00000018498
-3.04	tripartite motif-containing protein 16-like	ENSTRUT00000020028	ENSTRUG00000008011
-3.02	paxillin	ENSTRUT00000041535	ENSTRUG00000016190

Table 6: Top 40 down-regulated transcripts by fold-change (fc) in TTX administration group

Quantitative real-time PCR

Gene-specific primer sequences are summarized in Table 7.

Table 7: Gene specific primer sequences for quantitative real-time PCR

beta actin 1 (Acc. No. U37499.1) - forward	5′-CTCTTCCAGCCATCCTTCCTT-3′
beta actin 1 (Acc. No. U37499.1) - reverse	5′-GACGTCGCACTTCATGATGCT-3′
ENSTRUT00000045544 - forward	5′-GGCACCACACCTTCAATCCT-3′
ENSTRUT00000045544 - reverse	5′-GGCTGGGAACAGATGGAATG-3′

Real-time PCR analysis of mRNA expression of chymotrypsin-like elastase family member 2A transcript ENSTRUT00000045544 showed a significant expression level between the control group (1.00 ± 0.77) and the TTX administration group (27.22 ± 4.18) with a p-value of 0.0019. This result shows good correlation with the fold-change difference in expression of 37.6-fold, as measured by microarray analysis (Figure 4).

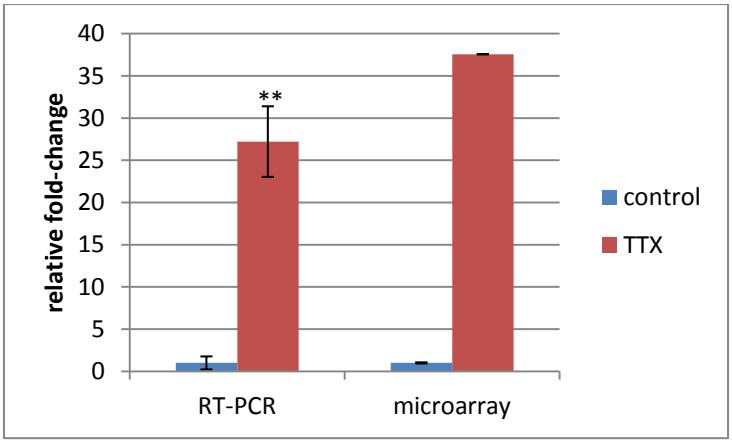


Figure 4: Relative fold-change difference in expression of transcript ENSTRUT00000045544 in the TTX administration group compared to nontoxic control group as measured by RT-PCR and microarray analysis; *** p < 0.01*

Discussion

By using an injection experiment with cultured pufferfish, we can control and equalize the surroundings in

which the fish are raised, and therefore neutralize individual differences in gene expression caused by environmental factors in a way that is impossible to do when using wild specimen as in section 1 of this dissertation. Another factor to keep in mind is, that since the cultured *T. rubripes* specimens used in this study were nontoxic prior to injection, only gene expression differences after 5 days of first exposure to TTX are reflected in the results, and short-term differences as well as long-term differences could well be different from what we observed.

Sampling

The specimens were all uniform in mass and size – a sign of their upbringing in the same environment. TTX recovery of 28±13% of the applied one-time dose can be considered high and confirms the expected accumulation potential observed in *T. rubripes*. Previous examination of [^{3}H] TTX in juvenile cultured *T. rubripes* revealed that 19% and 15.7% of the total radioactivity was accumulated in the liver, respectively, with the highest amount of 45.1% and 54.1% being found in the skin[73].

Differential gene expression

As with gene expression in wild pufferfish, more genes were found to be significantly down-regulated than up-regulated. GO annotation shows largely the same distribution as in section 1 with wild pufferfish, with two notable differences. First of all, the term "biological adhesion" is absent in the other experiments, but represented here by 12 transcripts. This term describes the adhesion of a cell to a substrate or organism. The second difference is the relatively high representation of the Molecular Function term "signal transducer activity" (41 transcripts), which suggests a higher prevalence of signal transduction related DEGs and an influence of TTX on signal transduction, likely acting via transcription factor SOX-5, nuclear receptor coactivator 2, and/or hepatocyte nuclear factor 3-gamma, which were all down-regulated in TTX administered fish.

KEGG pathway results are similar to those of genes in wild liver, with purine and thiamine metabolism related pathways being highly represented among the tested genes. Since thiamine is necessary for biosynthesis of neurotransmitters like acetylcholine and plays an important role in neural health, a difference

in its metabolism between toxic and nontoxic pufferfish is likely a response to TTX neurotoxicity. “T cell receptor signalling pathway” related transcripts were also enriched and suggest an effect of TTX on expression of immune system related genes.

The highest up-regulated gene after TTX administration was chymotrypsin-like elastase family 2A, also known as elastase 2A. This result was confirmed using quantitative real-time PCR, indicating validity of the microarray analysis experiment. The product of the homologous gene in humans is found to be secreted in pancreatic juice with an elastic and fibrous protein digesting function[74]. Recently, TTX secretion into bile has been demonstrated and quantified, suggesting a correlation to the secretion of elastase 2A[75].

The sodium channel beta-2 subunit gene was up-regulated after TTX administration with a fold-change difference of 4 compared to the control. This sodium channel subunit is reported to modulate mRNA and protein expression of TTX-sensitive voltage-gated sodium channels[76]. This highly suggests that TTX influences modulates and changes the expression of voltage-gated sodium channels via interaction with subunit beta-2.

Based on the up-regulation of Rho GTPase-activating protein 29 and up-regulation of the G-protein-coupled receptor 22 gene, the GTP-binding protein Rheb gene and Arf GAP with GTPase domain ankyrin repeat and the PH domain 2 gene, it seems plausible that a G-protein coupled receptor signal transduction system might be involved in the cellular response to TTX and its accumulation, metabolism or elimination.

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Supplementary Information

Modified Hank's balanced salt solution buffer:

Sodium chloride (NaCl)	160 mM
Potassium chloride (KCl)	5.4 mM
Disodium phosphate (Na_2HPO_4)	0.34 mM
Monopotassium phosphate (KH_2PO_4)	0.44 mM
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid	10 mM

(adjust to pH 7.4 with sodium hydroxide solution)