Transcriptome analysis of tetrodotoxin sensing and action of tetrodotoxin in central nervous system of tiger puffer *Takifugu rubripes* juveniles

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Abstract

To reveal tetrodotoxin (TTX) sensing and action of TTX in central nervous system (CNS) of tiger puffer *Takifugu rubripes* juveniles, we conducted transcriptome analysis by next-generation sequencing for the olfactory and the brain of non-toxic cultured juveniles which were sensed and administered TTX. Sixty seven million reads from the nasal region (olfactory epithelium and skin) and the brain of each of three individuals of the control, TTX-sensed and TTX-administered juveniles were assembled into 153,958 contigs. A mapping of raw reads from the each sample onto the nucleotide sequences of predicted transcripts in *T. rubripes* genome (FUGU version 4) and the de novo assembled contigs, conducted to investigate their frequency of expression, revealed that the expression of 21 and 81 known genes significantly changed in TTX-sensed and TTX-administered juveniles in comparison with control juveniles, respectively. These genes included those related to feeding regulation and reward system, indicate that TTX ingestion of *T. rubripes* juveniles is controlled at feeding center in brain and *T. rubripes* may sense TTX as a reward, and accumulated TTX will directly act on CNS to adjust TTX ingestion.

Keywords *Takifugu rubripes* • Tetrodotoxin (TTX) • Central nervous system • RNA-seq • Feeding center • Reward system

Introduction

Marine pufferfish of the genus *Takifugu* contain tetrodotoxin (TTX) which is one type of potent neurotoxin specific to voltage-gated sodium channels of excitable membranes of muscle and nerve tissues [1-3]. Matsumura [4] found that the toxin levels in embryos of grass puffer *Takifugu niphobles* increase from fertilization to hatching and concluded that TTX is produced by pufferfish. Other studies claimed that pufferfish accumulates TTX through food chain [3, 5], that is originally produced by marine bacteria belonging to the genera *Vibrio* and *Shewanella* [6-9]. The hypothesis that TTX in pufferfish is exogenous and is derived via the food chain is now widely accepted, because this hypothesis was supported by the fact that artificially raised tiger puffer *Takifugu rubripes* become non-toxic when fed with non-toxic diets in the environment where the invasion of TTX-bearing organisms was eliminated [10, 11], and such non-toxic *T. rubripes* are attracted to TTX [12, 13] and become toxic when they were fed with TTX-containing diets [14, 15, 16].

Non-toxic fishes can detect TTX at very low levels by gustatory organ [17]. Once non-toxic fishes ingest toxic eggs of pufferfish, they spit out pufferfish eggs immediately [18]. It was also confirmed that non-toxic fishes die even in trace amounts of TTX when administered directly into their bodies [19]. These evidences indicate that non-toxic fishes can recognize and avoid TTX as toxin. In contrast, *T. rubripes* detects TTX by olfactory organ, and actively ingests [13] and then accumulate high amounts of TTX [10]. Recently, several proteins implicated in the toxicity of pufferfish have been reported. Skeletal muscle voltage-gated Na^+ channel in pufferfish gain TTX resistance by amino acid substitutions in the P-loop region of the proteins [20-22]. Pufferfish saxitoxin and tetrodotoxin-binding proteins (PSTBPs) that bind to TTX and paralytic shellfish toxins were isolated from the plasma of panther puffer *Takifugu pardalis* and also found in the other *Takifugu* species [23, 24]. PSTBPs share high sequence homology (47 %) with a tributyltin-binding protein 2 (TBT-bp2) in Japanese flounder *Paralichthys olivaceus* [25], suggesting that PSTBPs originated in TBT-bp2s. These findings suggest that pufferfish become able to ingest TTX without recognizing as toxin through evolutional processes.

Generally liver and ovary of wild *T. rubripes* adults are strongly toxic [26]. However, in

73 juvenile stage, TTX is detected not only in liver but also in skin and brain of wild *T. rubripes* [16, 27].
74 It was further confirmed that TTX was transferred to skin and brain when TTX was administered to
75 cultured non-toxic *T. rubripes* juveniles [27]. Since predation is a major cause of mortality in *T.*
76 *rubripes* juveniles [28-30], bearing of TTX in skin may be functional as predator defense for the
77 juvenile pufferfish [16]. Therefore, pufferfish utilize TTX for its survival through evolutional
78 processes and alter the recognition of TTX as toxin for taking TTX into their body. Accumulation of
79 TTX in brain [27] suggests that TTX passed through blood-brain barrier and was transferred to the
80 central nervous system (CNS) of *T. rubripes* juveniles. Brain membranes of *T. pardalis* are harder to
81 bind to saxitoxin that has the same Na^+ channel blocking function as TTX than corresponding
82 membranes of rat same as skeletal muscle membranes including TTX-resistant Na^+ channel [20]. Thus,
83 TTX may be functional in brain of pufferfish without blocking Na^+ channel.
84 Given these evidences, we hypothesized that *T. rubripes* juvenile senses TTX as a
85 pharmacological agent and accumulated TTX is physiologically functional in CNS, and then some
86 changes occur in the expression of genes associated with TTX sensing and action of TTX in CNS.
87 Recently, next-generation sequencing technologies greatly improved the speed and efficiency of
88 transcriptome analysis in many organisms including fishes [31] and the availability of the whole
89 genome sequence of *T. rubripes* allowed us to use this technique. Thus, we conducted transcriptome
90 analysis by next-generation sequencing for the olfactory and the brain of non-toxic cultured *T. rubripes*
91 juveniles which were sensed and administered TTX.
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93 **Materials and methods**
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95 **Experimental fish**
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97 Non-toxic cultured *T. rubripes* juveniles (about 5 months old; body length, 11.0 ± 0.5 cm; body weight,
98 37.7 ± 4.1 g; n = 150) were purchased from a private hatchery (Tawaki Suisan Corp., Kumamoto,
99 Japan) and were transported to Research Center for Marine Invertebrates, National Research Institute

of Fisheries and Environment of Inland Sea, Fisheries Research Agency, Momoshima, Hiroshima, Japan, in July 2014. The fish were fed with the commercial diets (Otohime EP3, Marubeni Nissin Feed Co., Ltd., Tokyo, Japan) in an aerated 5,000-l tank until TTX treatment.

Purification of TTX

TTX was extracted from the ovary of a wild-caught adult *T. rubripes* according to the method of Ikeda et al. [32] with a slight modification. The extract was partially purified with Bio-Gel P-2 column (Bio-Rad Laboratories Inc., Herucles, CA, USA) and the absorbed TTX by the gel was eluted with 0.05 M acetic acid. TTX fraction was subjected to LC/MS analysis on an alliance LC/MS system equipped with a ZSpray MS 2000 detector (Waters, Milford, MA, USA) according to Nakashima et al. [33]. The amount of TTX (nanograms) determined by LC/MS was converted to mouse units (MU) based on the specific toxicity of TTX (220 ng/MU). Purified TTX was dried and frozen at -80°C until use.

TTX-sensing and TTX-administration treatment to *T. rubripes* juveniles

Preliminary tests [13, 27] elucidated that non-toxic cultured juveniles were generally attracted to TTX within 30 minutes of starting to smell TTX and intramuscularly administered TTX in the fish was transferred to brain at least 24 hours after administration [unpublished data]. Based on these results, the following methods were established. For TTX-sensing treatment, three non-toxic cultured juveniles were accommodated in an aerated 30 l tank filled with 20-l fresh sand filtered seawater for 30 minutes as control, and three other non-toxic juveniles were sensed to TTX by immersing 200 MU (44 μg) TTX-containing seawater during the same period. For TTX-administration treatment, 0.1 ml of saline (1.35 % NaCl) as control and 150 MU (33 μg) TTX solution dissolved with saline was administered in a single injection into the dorsal muscle of three other non-toxic cultured juveniles using a 1 ml disposable syringe (Terumo, Tokyo, Japan), and the both groups of juveniles were immediately returned to the 90-l tank. Then, all fish were collected at 24 hours after administration.

The control, TTX-sensed and TTX-administered juveniles were anesthetized on ice, and then nasal region (olfactory epithelium and skin) and brain tissues were sampled, and stored in RNA later (Qiagen, Valencia, CA, USA) at -80°C until use.

RNA extraction and cDNA library construction

Total RNA was extracted from the samples using RNeasy Mini Kit (Qiagen) following the manufacturer's instruction. The RNA samples were treated with DNase I (Takara, Tokyo, Japan) to digest contaminating genomic DNA. mRNA was then isolated from total RNA with Dynabeads® mRNA DIRECT™ Micro Kit (Life Technologies, Carlsbad, CA, USA). mRNA samples were fragmented, reverse transcribed and amplified to make barcoded whole transcriptome libraries using Ion Total RNA-seq Kit v2 (Life Technologies). Yield and size distribution of the fragmented RNA and the amplified cDNA were checked using an Agilent 2200 Tapestation with High Sensitivity RNA ScreenTape® and High Sensitivity D1000 ScreenTape® (Agilent Technologies, Palo Alto, CA, USA) at each step. We have performed a left size selection (< about 100 bp) with SPRIselect (Beckman Coulter, Krefeld, Germany) by using 1.2x volume of SPRI reagent to the nasal region samples. The average sizes of the amplified cDNAs were adjusted to be about 200 bp. Ion OneTouch™ System with Ion PI™ Template OT2 200 Kit v3 (Life Technologies) was used to prepare enriched, template-positive Ion PI™ Ion Sphere Particles.

Next-generation sequencing and data analysis

The cDNA libraries were sequenced with an Ion Proton™ System with an Ion PI™ Sequencing 200 Kit v3 (Life Technologies) following the manufacturer's instructions. Sequencing results were imported into CLC Genomic Workbench7.5 (CLC bio, Aarhus, Denmark) as FASTQ files for further analysis. On CLC Genomic Workbench, the raw reads with the quality score less than 0.05 were trimmed using the "Trim Sequences" tool. Reads shorter than 50 bp were discarded. De novo sequence

154 assembly was carried out on all trimmed reads from all libraries using the Trinity software [34] to
155 generate contigs. Duplicated and highly similar sequences were removed by the software CD-HIT (ver.
156 4.5.6. option, -c 0.9 [35]). Expression analysis was performed with RNA-seq Analysis Tool of CLC
157 Genomics Workbench for each library using the nucleotide sequences of predicted transcripts in *T.*
158 *rubripes* genome (FUGU version 4) cited from the Ensembl database and de novo assembled contigs
159 as references, respectively. Parameters for read mapping were set as follows: Length fraction 0.7,
160 similarity fraction 0.95. Gene expression was represented as RPKM (Reads Per Kilobase of exon
161 model per Million). Cluster analysis based on the RPKM was performed by CLUSTER3.0
162 (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm#ctv) using spearman correlation, and
163 Java TreeView (http://jtreeview.sourceforge.net/) was used to visualize clustering relationship.
164 Differential expression analysis between the control and TTX-sensed or TTX-administered juvenile
165 samples was performed using R version 2.15.2 software (R Development Core Team 2008) package
166 TCC with a false discovery rate (FDR) < 0.05 [36]. The homology searches of contigs detected as
167 differential expression genes (DEGs) were conducted using BLASTX (*e* value 1e-5) against the NCBI
168 non-redundant protein database. The DEGs assigned as unnamed protein products or uncharacterized
169 proteins were excluded and we called them “known DEGs” in this paper.
170
171 **Results**
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173 **Sequencing and de novo assembly of nasal region and brain tissue transcripts**
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175 Next-generation sequencing was conducted to generate expressed short reads from nasal region
176 (olfactory epithelium and skin) and brain of the control, TTX-sensed and TTX-administered *T.*
177 *rubripes* juveniles. We obtained 66,971,623 reads (2,192k – 3,325k reads/individual), with total
178 nucleotides of 7,167,786,900 bp (231M – 364M bp/individual) (Table 1). Based on the reads, 153,958
179 contigs, with an average length of 648 bp were assembled (Table 2).
180

Read mapping and gene annotation

The sequence reads were mapped to the nucleotide sequences of predicted transcripts in *T. rubripes* genome (FUGU version 4) cited from the Ensembl database and de novo assembled contigs to calculate the expression values. A hierarchical clustering analysis using the RNA-seq data analyzed by mapping to *T. rubripes* genome revealed that in Nasal region, the smaller and medium clusters tended to be form among samples (control and TTX) and between the trial groups (sensing and administration), respectively, and the larger clusters were formed between the tissues (nasal region and brain). However, the clusters were only formed about tissues by mapping to the constructed contigs (Fig. 1). The expression values were compared between the control and TTX-sensed or TTX-administered *T. rubripes* juveniles. The number of known DEGs detected under TTX-sensing treatment compared to the control were 4 (19.0 % of total number of DEGs) in nasal region and 17 (25.0 %) in brain, respectively (Table 3). In TTX-administration treatment, the number of known DEGs were 38 (37.3 %) in nasal region and 43 (35.0 %) in brain, respectively (Table 3).

Expressed genes for TTX-sensed or TTX-administered juveniles

The distinctly expressed known DEGs in nasal region of TTX-sensed juveniles showed no high (fold change (FC) > 10) and low (FC < -10) expression levels, while in brain, relatively high and low expression levels were observed in several genes such as those encoding long-chain-fatty-acid--CoA ligase 5-like (FC value of 78.04), hemoglobin embryonic subunit alpha-like (FC value of 61.93) and peptide yy-like (FC value of -14.42) (Table 4, 5). The known DEGs which showed relatively high and low expression levels were also detected under TTX-administration treatment. In nasal region, extracellular superoxide dismutase (FC value of 36.79), envelope polyprotein (FC value of 15.22), receptor (chemosensory) transporter protein 4 (FC value of 12.20), podocalyxin-like (FC value of -24.90), tRNA-splicing endonuclease subunit sen15-like (FC value of -21.11), nuclear fragile x mental retardation-interacting protein 1-like (FC value of -20.19), period homolog 3 (drosophila) (FC value

208 of -16.71) and integrin alpha-3-like (FC value of -12.78) were detected (Table 6). In brain, potassium
209 voltage-gated channel subfamily b member 2-like (FC value of 11.85), sorbin and sh3 domain-
210 containing protein 2-like (FC value of 10.09) and period homolog 3 (drosophila) (FC value of -10.26)
211 were detected (Table 7). In addition, several known DEGs were detected in both nasal region and brain
212 of *T. rubripes* juvenile, such as those encoding long-chain-fatty-acid--CoA ligase 5-like under TTX-
213 sensing treatment (Table 4, 5) and period homolog 3 (drosophila), envelope polyprotein, period
214 circadian protein homolog 2-like and lipocalin precursor under TTX-administration treatment (Table
215 6, 7), and vasoactive intestinal peptide (vip) were down-regulated in brain under both TTX-sensing
216 and TTX-administration treatment (Table 5, 7).
217
218 **Discussion**
219
220 In this study, we compared the gene expression in olfactory and brain among cultured *T. rubripes*
221 juveniles with or without TTX-sensing and TTX-administration by transcriptome analysis using next-
222 generation sequencing. Hierarchical cluster analysis of expressed genes was performed to assess the
223 transcriptional pattern variation. In the case of using the RNA-seq data analyzed by mapping to *T.*
224 *rubripes* genome revealed that in Nasal region, the smaller clusters tended to be form among samples
225 (control and TTX), but the medium clusters tended to be form between the trial groups (sensing and
226 administration) for each tissue. These results indicate that the gene expression in olfactory and brain
227 of *T. rubripes* juveniles was affected by the operation and was not dramatically changed by TTX
228 treatment. However, a number of DEGs detected under TTX-sensing and TTX-administration
229 treatment compared to the control. Based on these DEGs, the following shows TTX sensing and action
230 of TTX in CNS of *T. rubripes* juveniles.
231
232 **TTX sensing of *T. rubripes* juveniles**
233
234 In nasal region (olfactory epithelium and skin) of TTX-sensed juveniles, mitogen-activated protein 4

kinase 4-like isoform x2 gene that is inhibitor of adipogenesis [37] was highest up-regulated than the fresh seawater-immersed control juveniles. In addition, long-chain-fatty-acid--CoA ligase 5 (ACSL5)-like gene which plays role in triacylglycerol (TAG) synthesis [38, 39] was up-regulated by TTX-sensing. These results and evidences suggest that TTX-sensing affects lipid metabolism in nasal region of *T. rubripes* juveniles. However, the expression of genes related to olfaction did not change by TTX-sensing. Given that cultured *T. rubripes* has not encountered TTX-bearing organisms, *T. rubripes* may instinctively sense TTX.

In brain of TTX-sensed juveniles, ACSL5-like and hemoglobin embryonic subunit alpha-like genes were extremely up-regulated than control fish. ACSL5 that is involved in TAG synthesis [38, 39] was also highly expressed in nasal region of TTX-sensed juveniles, suggesting that TTX-sensing particularly affects lipid metabolism in nervous system. Highly expression of one kind of hemoglobin, which is involved in oxygen transport, suggests that nervous activity is promoted in brain of TTX-sensed *T. rubripes* juveniles. Peptide yy (PYY)-like gene that has an appetite-regulation effect on fish [40-42] was down-regulated by TTX-sensing. In addition, vip peptides-like and TPA_inf: tachykinin 1 genes which have a function as anorexigenic peptides in fishes [43, 44] were also down-regulated by TTX-sensing. Tachykinins is also related to dopaminergic system in mammals [45, 46]. In addition, Thy-1 membrane glycoprotein gene which may modulate dopamine metabolism in mammals [47] was down-regulated by TTX-sensing. If these evidences are applied to in fishes, some changes might occur in dopaminergic systems of TTX-sensed *T. rubripes* juveniles. Some studies have suggested the involvement of dopaminergic pathways in the central regulation of food intake in fishes [48-50]. Thus, TTX ingestion of *T. rubripes* juveniles is controlled at feeding center in brain and *T. rubripes* juveniles might sense TTX as a reward.

Action of TTX in CNS of *T. rubripes* juveniles

In nasal region of TTX-administered juveniles, extracellular superoxide dismutase gene, which protects the living body from oxidative stress, was highest up-regulated than saline-administered

control juveniles. This study demonstrated the up-regulation of receptor (chemosensory) transporter protein 4 (RTP4). RTP family members are probable chaperon protein which facilitates trafficking and functional cell surface expression of some G-protein coupled receptors such as odorant receptor [51] and bitter taste receptor [52], suggesting that RTP4 is expressed in olfactory epithelium by TTX-administration and acts as a transporting protein of TTX sensing receptor. Podocalyxin-like gene, which is known to be expressed in the developing brain of the mouse and plays multiple roles in neural development [53], was lowest down-regulated. In addition, this study demonstrated the up- and down-regulation of cyclin-dependent kinase inhibitor 1-like isoform x1 which associates with olfactory epithelium regeneration [54], and immunoglobulin superfamily member 8-like, which facilitates olfactory sensory synapse formation [55], respectively. In fishes, neurogenesis continues throughout life under the influence of environmental experience [56]. Synthesizing these results and evidences, we presume that nerve cell renewal occurs in the olfactory system of *T. rubripes* under the influence of TTX which exists in the olfactory epithelium. The expression of per genetic group which ticks in the center of cell clock [57] was specifically down-regulated by TTX-administration as following: period circadian protein homolog 1 -like isoform x1, period circadian protein homolog 2-like and period homolog 3 (Drosophila). These results suggest that biological rhythm of *T. rubripes* juveniles changed by accumulating TTX in their body. The core feedback loop of clock genes accurately ticks every 24 h [57]. Thus, there was another possibility that sampling times of juveniles was related to the clock genes expression.

In brain of TTX-administered juveniles, potassium voltage-gated channel subfamily b member 2-like gene, which mediates membrane hyperpolarization during trains of action potentials [58, 59], was highest up-regulated than control fish. In addition, the expression of some genes which may be related to release of neurotransmitters changed by TTX-administration as following: clathrin, light chain [60], SRC kinase signaling inhibitor 1-like [61] and synaptotagmin-c-like [62]. SRC kinase signaling inhibitor 1 is involved in the formation and maintenance of synapses during developmental processes of brain [61]. Further, protein phosphatase 1B-like which involves in neurodegeneration [63] was up-regulated by TTX-administration, respectively. There are at least two main forms of neural

289 plasticity; biochemical switching and structural reorganization [64, 65]. Neural plasticity aids in the
290 adaptation and flexibility demanded by the diverse environment in which fishes inhabit [66]. Non-
291 toxic cultured *T. rubripes* juveniles is inferior in fear response comparing to the toxic wild juveniles,
292 and release experiment into the pond with predators revealed that survival of cultured pufferfish with
293 no TTX was significantly lower than that of toxic wild juveniles [28, 29]. These evidences suggest
294 that *T. rubripes* juveniles utilize TTX to adapt to the environment with action of TTX in CNS. This
295 study demonstrated the down-regulation of lipocalin precursor by TTX-administration in both nasal
296 region and brain of *T. rubripes* juveniles. TBT-bp2 in the blood of *P. olivaceus* belongs to the lipocalin
297 superfamily and shows highly identity to PSTBPs of *T. pardalis* [25]. From the fact that *T. rubripes*
298 also have PSTBPs [24, 67, 68], the expression of lipocalin precursor may change in relation to the
299 accumulation of TTX in their body. Interestingly, in brain of *T. rubripes* juveniles, vip which have a
300 function as anorexigenic peptides in fish [44] were down-regulated by not only TTX-sensing but also
301 TTX-administration. It may interpret that action of TTX to feeding center is not limited to only at the
302 time of TTX ingestion, accumulated TTX also directly acts on CNS and adjust the intake.
303 In this study, we focused on the gene expression associated with TTX sensing and action of
304 TTX in CNS of *T. rubripes* juveniles, thus did not concern the specificity of the fish to TTX. In the
305 future, we need to use some other alkaloid such as palytoxin that is known for having other kinds of
306 pufferfish [69] to investigate whether gene expression, behavioral and physiological change of *T.*
307 *rubripes* juveniles are specific to TTX.
308
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316 **References**

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318 1. Colquhon D, Henderson R, Ritchie JM (1972) The binding of labelled tetrodotoxin to non-
319 myelinated nerve fibres. J Physiol 227: 95-126

320 2. Narahashi T (2001) Pharmacology of tetrodotoxin. J Toxicol Toxin Rev 20: 67-84

321 3. Noguchi T, Arakawa O, Takatani T (2006) TTX accumulation in pufferfish. Comp Biochem
322 Physiol D1: 145-152

323 4. Matsumura K (1998) Production of tetrodotoxin in puffer fish embryos. Environ Toxicol Phar 6:
324 217-219

325 5. Yasumoto T, Yotsu-Yamashita M (1996) Chemical and etiological studies on tetrodotoxin and its
326 analogs. J Toxicol-Toxin Rev 15: 81-90

327 6. Noguchi T, Jeon JK, Arakawa O, Sugita H, Deguchi Y, Shida Y, Hashimoto K (1986) Occurrence
328 of tetrodotoxin in *Vibrio* sp. isolated from the intestines of a xanthid crab, *Atergatis floridus*. J
329 Biochem 99: 311-314

330 7. Yasumoto T, Yasumura D, Yotsu M, Michishita T, Endo A, Kotaki Y (1986) Bacterial production
331 of tetrodotoxin and anhydrotetrodotoxin. Agric Biol Chem 50: 793-795

332 8. Narita H, Matsubara S, Miwa N, Akahane S, Murakami M, Goto T, Nara M, Noguchi T, Saito T,
333 Shida Y, Hashimoto K (1987) *Vibrio alginolyticus*, a TTX-producing bacterium isolated from the
334 starfish *Astropecten polyacanthus*. Nippon Suisan Gakkaishi 47: 935-941

335 9. Matsui T, Taketsugu S, Sato H, Yamamori K, Kodama K, Ishii A, Hirose H, Shimizu C (1990)
336 Toxification of cultured puffer fish by the administration of tetrodotoxin producing bacteria.
337 Nippon Suisan Gakkaishi 56: 705

338 10. Saito T, Maruyama J, Kanoh S, Jeon JK, Noguchi T, Harada T, Murata O, Hashimoto K (1984)
339 Toxicity of the cultured pufferfish *Fugu rubripes rubripes* along with their resistibility against
340 tetrodotoxin. Nippon Suisan Gakkaishi 50: 1573-1575 (**in Japanese**)

341 11. Noguchi T, Arakawa O, Takatani T (2006) Toxicity of pufferfish *Takifugu rubripes* cultured in
342 netcages at the sea or aquaria on land. Comp Biochem Physiol D1: 153-157

12. Saito T, Kageyu K, Goto H, Murakami N, Noguchi T (2000) Tetrodotoxin attracts pufferfish ("torafugu" *Takifugu rubripes*). Bull Inst Oceanic Res & Develop Tokai Univ 21: 93-96
13. Okita K, Yamazaki H, Sakiyama K, Yamane H, Niina S, Takatani T, Arakawa O, Sakakura Y (2013) Puffer smells tetrodotoxin. Ichthyol Res 60: 386-389
14. Matsui T, Hamada S, Konosu S (1981) Difference in accumulation of puffer fish toxin and crystalline tetrodotoxin in the puffer fish, *Fugu rubripes rubripes*. Nippon Suisan Gakkaishi 47: 535-537
15. Honda S, Arakawa O, Takatani T, Tachibana K, Yagi M, Tanigawa A, Noguchi T (2005) Toxification of cultured puffer fish *Takifugu rubripes* by feeding on tetrodotoxin-containing diet. Nippon Suisan Gakkaishi 71: 815-820 (**in Japanese**)
16. Sakakura Y, Takatani T, Nakayasu J, Yamazaki H, Sakiyama K (2016) Administration of tetrodotoxin protects artificially raised juvenile tiger puffer *Takifugu rubripes* from predators. Fish Sci. doi: 10.1007/s12562-016-1046-0
17. Yamamori K, Nakamura M, Matsui T, Hara TJ (1988) Gustatory response to tetrodotoxin and saxitoxin in fish: a possible mechanism for avoiding marine toxins. Can J Fish Aquat Sci 45: 2182-2186
18. Itoi S, Yoshikawa S, Asahina K, Suzuki M, Ishizuka K, Takimoto N, Mitsuoka R, Yokoyama N, Detake A, Takayanagi C, Eguchi M, Tatsuno R, Kawane M, Kokubo S, Takanashi S, Miura A, Suitoh K, Takatani T, Arakawa O, Sakakura Y, Sugita H (2014) Larval pufferfish protected by maternal tetrodotoxin. Toxicon 78: 35-40
19. Saito T, Noguchi T, Harada T, Murata O, Abe T, Hashimoto K (1985) Resistibility of toxic and nontoxic pufferfish against tetrodotoxin. Nippon Suisan Gakkaishi 51: 1371
20. Yotsu-Yamashita M, Nishimori K, Nitanai Y, Isemura M, Sugimoto A, Yasumoto T (2000) Binding properties of ^{3}H-PbTx-3 and ^{3}H-saxitoxin to brain membranes and to skeletal muscle membranes of puffer fish *Fugu pardalis* and the primary structure of a voltage-gated Na^{+} channel α-subunit (fMNa1) from skeletal muscle of *F. pardalis*. Biochem Biophys Res Commun 267: 403-412

21. Venkatesh B, Lu SQ, Dandona N, See SL, Brenneer S, Soong TW (2005) Genetic basis of tetrodotoxin resistance in pufferfishes. Curr Biol 15: 2069-2072
22. Maruta S, Yamaoka K, Yotsu-Yamashita M (2008) Two critical residues in p-loop regions of puffer fish Na^+ channels on TTX sensitivity. Toxicon 51: 381-387
23. Yotsu-Yamashita M, Sugimoto A, Terakawa T, Shoji Y, Miyazawa T, Yasumoto T (2001) Purification, characterization, and cDNA cloning of a novel soluble saxitoxin and tetrodotoxin binding protein from plasma of the puffer fish, *Fugu pardalis*. Eur J Biochem 268: 5937-5946
24. Yotsu-Yamashita M, Yamaki H, Okoshi N, Araki N (2010) Distribution of homologous proteins to puffer fish saxitoxin and tetrodotoxin binding protein in the plasma of puffer fish and among the tissues of *Fugu pardalis* examined by Western blot analysis. Toxicon 55: 1119-1124
25. Oba Y, Shimasaki Y, Oshima Y, Satone H, Kitano T, Nakao M, Kawabata S, Honjo T (2007) Purification and characterization of tributyltin-binding protein type 2 from plasma of Japanese flounder, *Paralichthys olivaceus*. J Biochem 142: 229-238
26. Noguchi T, Arakawa O (2008) Tetrodotoxin – distriburion and accumulation in aquatic organisms, and cases of human intoxication. Mar Drugs 6: 220-242
27. Okita K, Takatani T, Nakayasu J, Yamazaki H, Sakiyama K, Ikeda K, Arakawa O, Sakakura Y (2013) Comparison of the localization of tetrodotoxin between wild pufferfish *Takifugu rubripes* juveniles and hatchery-reared juveniles with tetrodotoxin administration. Toxicon 71: 128-133
28. Shimizu D, Sakiyama K, Sakakura Y, Takatani T, Takahashi Y (2007) Predation differences between wild and hatchery-reared tiger puffer *Takifugu rubripes* juveniles in a salt pond mesocosm. Nippon Suisan Gakkaishi 73: 461-469 (**in Japanese**)
29. Shimizu D, Sakiyama K, Sakakura Y, Takatani T, Takahashi Y (2008) Quantitative evaluation of post-release mortality using salt pond mesocosm: case studies of hatchery and wild juvenile tiger puffer. Rev Fish Sci 16: 195-203
30. Nakajima H, Kai M, Koizumi K, Tanaka T, Machida M (2008) Optimal release locations of juvenile ocellate puffer *Takifugu rubripes* identified by tag and release experiments. Rev Fish Sci 16: 228-234

31. Sato Y, Hachiya T, Iwasaki W (2012) Next-generation sequencing in aquatic biology: current status and future directions. Fish Genet Breed Science 41: 17-32 (**in Japanese**)
32. Ikeda K, Murakami Y, Emoto Y, Ngy L, Taniyama S, Yagi M, Takatani T, Arakawa O (2009) Transfer profile of intramuscularly administered tetrodotoxin to non-toxic cultured specimens of the pufferfish *Takifugu rubripes*. Toxicon 53: 99-103
33. Nakashima K, Arakawa O, Taniyama S, Nonaka M, Takatani T, Yamamori K, Fuchi Y, Noguchi T (2004) Occurrence of saxitoxins as a major toxin in the ovary of a marine puffer *Arothron firmamentum*. Toxicon 43: 207-212
34. Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, Adiconis X, Fan L, Raychowdhury R, Zeng Q, Chen Z, Mauceli E, Hacohen N, Gnirke A, Rhind N, di Palma F, Birren BW, Nusbaum C, Lindblad-Toh K, Friedman N, Regev A (2011) Full-length transcriptome assembly from RNA-seq data without a reference genome. Nature Biotech 29: 644-652
35. Li W, Godzik A (2006) Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. Bioinformatics 22: 1658-1659
36. Sun J, Nishiyama T, Shimizu K, Kadota K (2013) TCC: an R package for comparing tag count data with robust normalization strategies. BMC Bioinfo 14: 219
37. Tang X, Guilherme A, Chakladar A, Powelka AM, Konda S, Virbasius JV, Nicoloro SM, Straubhaar J, Czech MP (2006) An RNA interference-based screen identifies MAP4K4/NIK as a negative regulator of PPARγ, adipogenesis, and insulin-responsive hexose transport. Proc Natl Acad Sci USA 103: 2087-2092
38. Horton JD, Shah NA, Warrington JA, Anderson NN, Park SW, Brown MS, Goldstein JL (2003) Combined analysis of oligonucleotide microarray data from transgenic and knockout mice identifies direct SREBP target genes. Proc Natl Acad Sci USA 100: 12027-12032
39. Achouri Y, Hegarty BD, Allanic D, Becard D, Hainault I, Ferre P, Foufelle F (2005) Long chain fatty acyl-CoA synthetase 5 expression is induced by insulin and glucose: involvement of sterol regulatory element-binding protein-1c. Biochimie 87: 1149-1155
40. Gonzalez R, Unniappan S (2010) Molecular characterization, appetite regulatory effects and

424 feeding related changes of peptide YY in goldfish. Gen Comp Endocrinol 166: 273-279
425 41. Chen Y, Shen Y, Pandit NP, Fu J, Li D, Li J (2013) Molecular cloning, expression analysis, and
426 potential food intake attenuation effect of peptide YY in grass carp (*Ctenopharyngodon idellus*).
427 Gen Comp Endocrinol 187: 66-73
428 42. Chen Y, Pandit NP, Fu J, Li D, Li J (2014) Identification, characterization and feeding response
429 of peptide YYb (PYYb) gene in grass carp (*Ctenopharyngodon idellus*). Fish Physiol Biochem
430 40: 45-55
431 43. Peyon P, Saied H, Lin X, Peter RE (2000) Preprotachykinin gene expression in goldfish brain:
432 sexual, seasonal, and postprandial variations. Peptides 21: 225-231
433 44. Matsuda K, Maruyama K (2007) Regulation of feeding behavior by pituitary adenylate cyclase-
434 activating polypeptide (PACAP) and vasoactive intestinal polypeptide (VIP) in vertebrates.
435 Peptides 28: 1761-1766
436 45. Glowinski J, Kemel ML, Desban M, Gauchy C, Lavielle S, Chassaing G, Beaujouan JC,
437 Tremblay L (1993) Distinct presynaptic control of dopamine release in striosomal- and matrix-
438 enriched areas of the rat striatum by selective agonists of NK1, NK2 and NK3 tachykinin
439 receptors. Regul Pept 46: 124-128
440 46. Marco N, Thirion A, Mons G, Bougault I, Le Fur G, Soubrie P, Steinberg R (1998) Activation of
441 dopaminergic and cholinergic neurotransmission by tachykinin NK_3 receptor stimulation: an in
442 vivo microdialysis approach in guinea pig. Neuropeptides 32: 481-488
443 47. Conner JR, Wang XS, Neely EB, Ponnuru P, Morita H, Beard J (2008) Comparative study of the
444 influence of Thy1 deficiency and dietary iron deficiency on dopaminergic profiles in the mouse
445 striatum. J Neurosci Res 86: 3194-3202
446 48. De Pedro N, Delgado MJ, Pinillos ML, Alonso-Bedate M (1998) Alpha1-adrenergic and
447 dopaminergic receptors are involved in the anorectic effect of corticotropin-releasing factor in
448 goldfish. Life Sci 62: 1801-1808
449 49. Siddhuraju P, Becker K (2002) Effect of phenolic nonprotein amino acid L-dopa (L-3,4-
450 dihydroxyphenylalanine) on growth performance, metabolic rates and feed nutrient utilization of

451 common carp (*Cyprinus carpio* L.). Aquacult Nutr 6: 69-77
452 50. Johansson V, Winberg S, Björnsson BT (2005) Growth hormone-induced stimulation of
453 swimming and feeding behaviour of rainbow trout is abolished by the D_1 dopamine antagonist
454 SCH23390. Gen Comp Endocrinol 141: 58-65
455 51. Saito H, Kubota M, Roberts RW, Chi Q, Matsunami H (2004) RTP family members induce
456 functional expression of mammalian odorant receptors. Cell 119: 679-691
457 52. Behrens M, Bartelt J, Reichling C, Winnig M, Kuhn C, Meyerhof W (2006) Members of RTP
458 and REEP gene families influence functional bitter taste receptor expression. J Biol Chem 281:
459 20650-20659
460 53. Vitureira N, Andres R, Perez-Martinez E, Martinez A, Bribian A, Blasi J, Chelliah S, Lopez-
461 Domenech G, De Castro F, Burgaya F, McNagny K, Soriano E (2010) Podocalyxin is a novel
462 polysialylated neural adhesion protein with multiple roles in neural development and synapse
463 formation. PLoS One 5: e12003
464 54. Watabe-Rudolph M, Begus-Nahrmann Y, Lechel A, Rolyan H, Scheithauer M, Rettinger G, Thal
465 DR, Rudolph KL (2011) Telomere shortening impairs regeneration of the olfactory epithelium in
466 response to injury but not under homeostatic conditions. PLoS One 6: e27801
467 55. Ray A, Treloar HB (2012) IgSF8: A developmentally and functionally regulated cell adhesion
468 molecule in olfactory sensory neuron axons and synapses. Mol Cell Neurosci 50: 238-249
469 56. Zupanc GKH (2008) Adult neurogenesis and neuronal regeneration in the brain of teleost fish. J
470 Physiol Paris 102: 357-373
471 57. Okamura H, Doi M, Fustin JM, Yamaguchi Y, Matsuo M (2010) Mammalian circadian clock
472 system: molecular mechanisms for pharmaceutical and medical sciences. Adv Drug Deliv Rev
473 62: 876-884
474 58. Malin SA, Nerbonne J (2002) Delayed rectifier K^+ currents, IK, are encoded by Kv2 alpha-
475 subunits and regulate tonic firing in mammalian sympathetic neurons. J Neurosci 22: 10094-
476 10105
477 59. Johnston J, Griffin SJ, Baker C, Skrzypiec A, Chernova T, Forsythe ID (2008) Initial segment

478 Kv2.2 channels mediate a slow delayed rectifier and maintain high frequency action potential
479 firing in medial nucleus of the trapezoid body neurons. J Physiol 586: 3493-3509
480 60. Granseth B, Odermatt B, Royle S, Lagnado L (2006) Clathrin-mediated endocytosis is the
481 dominant mechanism of vesicle retrieval at hippocampal synapses. Neuron 51: 773-786
482 61. Ito H, Atsuzawa K, Sudo K, Di Stefano P, Iwamoto I, Morishita R, Takei S, Semba R, Defilippi
483 P, Asano T, Usuda N, Nagata K (2008) Characterization of a multidomain adaptor protein,
484 p140Cap, as part of a pre-synaptic complex. J Neurochem 107: 61-72
485 62. Sugita S, Han W, Butz S, Liu X, Fernandez-Chacon R, Lao Y, Südhof TC (2001) Synaptotagmin
486 VII as a plasma membrane Ca2+ sensor in exocytosis. Neuron 30: 459-473
487 63. Klumpp S, Selke D, Ahlemeyer B, Schaper C, Krieglstein J (2002) Relationship between protein
488 phosphatase type-2C activity and induction of apoptosis in cultured neuronal cells. Neurochem
489 Int 41: 251-259
490 64. Zupanc GKH, Lamprecht J (2000) Towards a cellular understanding of motivation: structural
491 reorganization and biochemical switching as key mechanisms of behavioral plasticity. Ethology
492 106: 467-477
493 65. Oliveira RF (2009) Social behavior in context: hormonal modulation of behavioral plasticity and
494 social competence. Integr Comp Biol 49: 423-440
495 66. Gonda A, Herczeg G, Merilä J (2011) Population variation in brain size of nine-spined
496 sticklebacks (*Pungitius pungitius*) – local adaptation or environmentally induced variation? BMC
497 Evol Biol 11: 75
498 67, Tatsuno R, Yamaguchi K, Takatani T, Arakawa O (2013) RT-PCR- and MALDI-TOF mass
499 spectrometry-based identification and discrimination of isoforms homologous to pufferfish
500 saxitoxin- and tetrodotoxin-binding protein in the plasma of non-toxic cultured pufferfish
501 (*Takifugu rubripes*). Biosci Biotechnol Biochem 77: 208-212
502 68. Hashiguchi Y, Lee JM, Shiraishi M, Komatsu S, Miki S, Shimasaki Y, Mochioka N, Kusakabe T,
503 Oshima Y (2015) Characterization and evolutionary analysis of tributyltin-binding protein and
504 pufferfish saxitoxin and tetrodotoxin-binding protein genes in toxic and nontoxic pufferfishes. J

505 Evol Biol 28: 1103-1118

506 69. Taniyama S, Mahmud Y, Tanu MB, Takatani T, Arakawa O, Noguchi T (2001) Delayed

507 haemolytic activity by the freshwater puffer *Tetraodon* sp. toxin. Toxicon 39: 725-727

Tables

Table 1 Overview of the sequencing of cDNA from nasal region (olfactory epithelium and skin) and brain of TTX-sensed and TTX-administered *Takifugu rubripes* juveniles

Items	TTX-sensing treatment				TTX-administration treatment			
	Nasal region		Brain		Nasal region		Brain	
	Control	TTX	Control	TTX	Control	TTX	Control	TTX
Total number of reads	2,587k ± 94k[a]	3,325k ± 829k	2,192k ± 197k	2,551k ± 117k	3,073k ± 406k	3,052k ± 386k	2,870k ± 439k	2,675k ± 191k
Total nucleotide length (bp)	261M ± 12M	355M ± 77M	231M ± 17M	269M ± 9M	364M ± 51M	337M ± 28M	299M ± 36M	273M ± 20M

[a] Results are shown as mean ± SD of 3 fish

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Table 2 Summary of de novo assembly of contigs from sequence reads for nasal region (olfactory epithelium and skin) and brain of TTX-sensed and TTX-administered *Takifugu rubripes* juveniles

Items	Number
Total number of reads	66,971,623
Total nucleotide length (bp)	7,167,786,900
Total length of contigs (bp)	99,781,233
Number of contigs	153,958
Longest contig (bp)	17,962
Average length (bp)	648

Table 3 Number of differential expression genes (DEGs) detected in nasal region (olfactory epithelium and skin) and brain of TTX-sensed and TTX-administered *Takifugu rubripes* juveniles

Mapping reference	Items	TTX-sensing treatment				TTX-administration treatment			
		Nasal region		Brain		Nasal region		Brain	
		Up-regulation	Down-regulation	Up-regulation	Down-regulation	Up-regulation	Down-regulation	Up-regulation	Down-regulation
T. rubripes genome	Total Number of DEGs	0	0	3	1	2	7	5	8
	Number of known DEGs	0	0	2	1	1	6	3	6
Contigs	Total Number of DEGs	14	7	31	33	48	45	50	60
	Number of known DEGs	3	1	5	9	14	17	18	16

Table 4 Genes that were up- and down-regulate in nasal region (olfactory epithelium and skin) of TTX-sensed *Takifugu rubripes* juveniles analyzed by mapping to contigs (FDR-corrected *p*-value <0.05)

Contig ID	Gene	Expression in RPKM[a] (mean ± SD, n=3)		Fold change
		Control	TTX	
c58136_g1_i1	Mitogen-activated protein 4 kinase 4-like isoform x2	21,892 ± 20,896	135,241 ± 65,074	6.18
c61343_g1_i2	Calpain-1 catalytic subunit-like	265,065 ± 78,263	442,603 ± 8,410	1.67
c73927_g1_i2	Long-chain-fatty-acid--CoA ligase 5-like	ND[b]	120,695 ± 38,720	NA[c]
c81231_g1_i3	Bromodomain-containing protein 3-like isoform x1	319,451 ± 48,324	65,555 ± 27,773	-4.87

[a] RPKM: reads per kilobase of exon model per million mapped reads

[b] ND: not detected

[c] NA: not applicable

Table 5 Genes that were up- and down-regulated in brain of TTX-sensed *Takifugu rubripes* juveniles analyzed by mapping to *T. rubripes* genome and contigs (FDR-corrected *p*-value <0.05)

ID	Gene	Expression in RPKM[a] (mean ± SD, n=3)		Fold change
		Control	TTX	
Differential expression genes (DEGs) detected by mapping to T. rubripes genome (Ensembl ID)				
ENSTRUT00000043560	Spermine synthase	9.3 ± 1.4	39.8 ± 11.6	4.28
ENSTRUT00000046894	Centromere protein N	ND[b]	9.2 ± 4.7	NA[c]
ENSTRUT00000047470	Peripherin	69.6 ± 30.9	11.2 ± 3.6	-6.19
Differential expression genes (DEGs) detected by mapping to contigs (Contig ID)				
c73927_g1_i2	Long-chain-fatty-acid-- CoA ligase 5-like	233.5 ± 404.4	18,221.6 ± 8,607.3	78.04
c51638_g1_i1	Hemoglobin embryonic subunit alpha-like	426.0 ± 737.9	26,383.3 ± 22,612.7	61.93
c71980_g1_i2	NADH dehydrogenase	14,183.9 ± 6,567.4	41,755.4 ± 22,042.0	2.94
c95098_g2_i1	Zinc finger protein Eos	48,870.1 ± 27,875.8	139,364.0 ± 11,130.5	2.85
c80686_g1_i1	General transcription factor IIF subunit 1-like	19,245.8 ± 2,027.7	47,290.7 ± 11,002.6	2.46
c70981_g1_i1	Peptide yy-like	8,298.1 ± 3,566.7	575.3 ± 996.5	-14.42
c78080_g1_i1	Urotensin ii-related peptide precursor	24,257.1 ± 8,487.6	3,352.4 ± 2,607.7	-7.24
c87450_g1_i1	Vip peptides-like	25,643.6 ± 16,773.2	4,403.0 ± 2,267.9	-5.82
c47673_g1_i1	Ras-related protein rab-8b-like	10,444.2 ± 4,183.4	2,020.7 ± 2,096.5	-5.17
c61457_g1_i1	Fibroblast growth factor receptor substrate 2-like	189,326.3 ± 94,323.7	77,067.8 ± 20,944.3	-2.46
c79330_g1_i1	Thy-1 membrane glycoprotein	242,220.3 ± 71,740.0	131,042.2 ± 26,743.4	-1.85
c90268_g1_i1	TPA_inf: tachykinin 1	97,141.8 ± 17,982.3	69,015.0 ± 5,749.7	-1.41
c90791_g1_i1	Neurobeachin-like isoform x3	59,884.0 ± 35,206.2	59,172.9 ± 22,897.8	-1.01
c75435_g1_i1	Growth hormone	114,851.9 ± 198,929.4	ND	NA

[a] RPKM: reads per kilobase of exon model per million mapped reads

[b] ND: not detected

[c] NA: not applicable

Table 6 Genes that were up- and down-regulated in nasal region (olfactory epithelium and skin) of TTX-administered *Takifugu rubripes* juveniles analyzed by mapping to *T. rubripes* genome and contigs (FDR-corrected *p*-value <0.05)

ID	Gene	Expression in RPKM[a] (mean ± SD, n=3)		Fold change
		Control	TTX	
Differential expression genes (DEGs) detected by mapping to T. rubripes genome				
(Ensembl ID)				
ENSTRUT00000016957	Receptor (chemosensory) transporter protein 4	1.1 ± 1.1	13.7 ± 2.8	12.20
ENSTRUT00000046106	Podocalyxin-like	20.3 ± 16.7	0.8 ± 1.4	-24.90
ENSTRUT00000007585	Period homolog 3 (Drosophila)	42.4 ± 21.3	2.5 ± 1.7	-16.71
ENSTRUT00000007590	Period homolog 3 (Drosophila)	28.9 ± 6.2	3.7 ± 2.8	-7.74
ENSTRUT00000003686	Complement component 8, gamma polypeptide	1,914.5 ± 723.9	463.4 ± 148.9	-4.13
ENSTRUT00000023711	Tubulin, alpha 2	112.8 ± 29.6	29.4 ± 2.3	-3.83
ENSTRUT00000038281	Circadian associated repressor of transcription	14.4 ± 10.5	ND[b]	NA[c]
Differential expression genes (DEGs) detected by mapping to contigs				
(Contig ID)				
c63197_g1_i1	Extracellular superoxide dismutase	60.6 ± 105.0	2,229.8 ± 1,997.3	36.79
c87942_g1_i5	Envelope polyprotein	108.8 ± 127.7	1,655.2 ± 145.6	15.22
c77064_g1_i2	Cyclin-dependent kinase inhibitor 1-like isoform x1	761.1 ± 397.1	5,517.0 ± 2,584.5	7.25
c79561_g1_i2	Cytoplasmic dynein 1 intermediate chain 2-like isoform x3	1,283.2 ± 89.2	7,521.1 ± 6,305.5	5.86
c62397_g1_i1	Protein inscuteable homolog	481.7 ± 261.8	2,723.7 ± 816.7	5.65
c62397_g1_i1	Diamine acetyltransferase 1-like	10,569.0 ± 693.0	29,130.7 ± 6,054.7	2.76
c81702_g1_i1	Double stranded rna-activated protein kinase 2	1,551.9 ± 1,704.3	3,931.7 ± 1,071.3	2.53
c85001_g1_i7	Tumor necrosis factor receptor superfamily member 4-like	5,622.7 ± 1,286.2	13,152.5 ± 3,530.8	2.34
c77678_g1_i2	Mannose-specific lectin-like	194,004.1 ± 15,127.9	362,323.5 ± 41,810.5	1.87
c77678_g1_i1	Lily-type lectin	104,210.6 ± 7,187.5	194,205.6 ± 10,327.1	1.86
c77678_g1_i5	Mannose-specific lectin-like	91,364.8 ± 12,267.0	166,608.0 ± 6,407.8	1.82
c90536_g2_i1	Ribonucleoside-diphosphate reductase subunit m2-like isoform x1	4,292.3 ± 1,508.3	7,823.3 ± 2,087.8	1.82
c91998_g1_i4	Apoptosis facilitator bcl-2-like protein 14	2,139.7 ± 586.8	3,790.4 ± 1,082.3	1.77
c62456_g1_i1	Serine threonine-protein kinase psk2	ND	3,232.8 ± 4,607.5	NA
c60649_g1_i1	tRNA-splicing endonuclease subunit sen15-like	1,649.6 ± 862.9	78.2 ± 135.4	-21.11
c59514_g1_i1	Nuclear fragile x mental retardation-interacting protein 1-like	2,010.7 ± 1,146.2	99.6 ± 172.5	-20.19
c89300_g1_i3	Integrin alpha-3-like	1,409.7 ± 255.1	110.3 ± 191.0	-12.78
c55712_g1_i1	Isoleucine--trna cytoplasmic-like	1,610.1 ± 250.9	172.2 ± 149.5	-9.35
c63419_g1_i3	Lysyl oxidase	2,177.1 ± 310.6	233.7 ± 202.9	-9.31
c89139_g2_i1	Protein capicua homolog isoform x3	1,901.6 ± 920.7	216.6 ± 192.3	-8.78
c91950_g1_i1	Period circadian protein homolog 2-like	9,901.5 ± 860.0	1,685.4 ± 919.8	-5.88
c15805_g1_i1	Immunoglobulin superfamily member 8-like	11,099.7 ± 8,539.1	2,290.3 ± 573.9	-4.85
c59867_g1_i1	Salivary glue protein	7,503.1 ± 2,447.5	1,745.9 ± 395.5	-4.30
c52572_g1_i1	Polyhomeotic-like protein 3-like isoform x3	2,979.2 ± 585.1	749.2 ± 408.3	-3.98
c84319_g1_i3	SEC14-like protein 2-like	3,540.0 ± 959.1	981.0 ± 719.3	-3.61
c95794_g1_i2	C-terminal binding protein 1	4,728.5 ± 1,069.8	1,509.4 ± 581.8	-3.13
c80106_g2_i1	Lipocalin precursor	179,726.2 ± 53,330.8	62,486.5 ± 20,641.1	-2.88
c94326_g5_i1	Elongation of very long chain fatty acids protein 6-like	2,132.0 ± 984.2	749.9 ± 303.0	-2.84
c85958_g2_i1	Cytochrome c oxidase subunit ii	773,930.0 ± 36,745.0	641,615.3 ± 51,394.4	-1.21
c62014_g1_i1	LIM domain and actin-binding protein 1-like	7,473.6 ± 6,187.9	6,769.8 ± 2,706.2	-1.10

c82952_g1_i2	FERM and PDZ domain-containing protein 1-like isoform x1	1,580.8 ± 1,410.4	ND	NA

[a] RPKM: reads per kilobase of exon model per million mapped reads

[b] ND: not detected

[c] NA: not applicable

Table 7 Genes that were up- and down-regulated in brain of TTX-administered *Takifugu rubripes* juveniles analyzed by mapping to *T. rubripes* genome and contigs (FDR-corrected *p*-value <0.05)

ID	Gene	Expression in RPKM[a] (mean ± SD, n=3)		Fold change
		Control	TTX	
Differential expression genes (DEGs) detected by mapping to T. rubripes genome				
(Ensembl ID)				
ENSTRUT00000043847	LIM domain only 2 (rhombotin-like 1)	7.9 ± 3.1	43.4 ± 15.4	5.48
ENSTRUT00000039938	Clathrin, light chain (Lca)	6.8 ± 2.3	35.7 ± 13.3	5.24
ENSTRUT00000006268	Family with sequence similarity 192, member A	ND[b]	7.2 ± 1.2	NA[c]
ENSTRUT00000007585	Period homolog 3 (Drosophila)	27.4 ± 4.3	2.7 ± 1.3	-10.26
ENSTRUT00000043060	NHP2 non-histone chromosome protein 2-like 1b (*Saccharomyces cerevisiae*)	25.4 ± 19.0	2.9 ± 1.1	-8.65
ENSTRUT00000022965	Pterin-4 alpha-carbinolamine dehydratase/dimerization cofactor of hepatocyte nuclear factor 1 alpha (TCF1) 2	28.6 ± 19.6	3.5 ± 2.3	-8.07
ENSTRUT00000008208	Immunoglobulin heavy variable 1-4	40.8 ± 24.0	7.9 ± 2.2	-5.14
ENSTRUT00000044170	Cytochrome P450, family 27, subfamily C, polypeptide 1	29.2 ± 4.9	8.2 ± 3.3	-3.56
ENSTRUT00000002671	Vasoactive intestinal peptide	16.6 ± 19.5	ND	NA
Differential expression genes (DEGs) detected by mapping to contigs				
(Contig ID)				
c33428_g1_i1	Potassium voltage-gated channel subfamily b member 2-like	91.3 ± 31.2	1,081.1 ± 598.5	11.85
c50125_g1_i1	Sorbin and sh3 domain-containing protein 2-like	92.2 ± 80.6	930.5 ± 105.7	10.09
c61302_g1_i1	Transmembrane protein 119-like	100.1 ± 109.9	918.0 ± 464.2	9.17
c33376_g1_i1	Ubiquitin-conjugating enzyme e2 o-like	148.6 ± 156.4	1,238.8 ± 442.8	8.34
c87942_g1_i5	Envelope polyprotein	289.5 ± 107.9	2,169.1 ± 76.3	7.49
c10845_g1_i1	Ankyrin repeat and sterile alpha motif domain containing 1b	231.3 ± 205.0	1,700.9 ± 1015.0	7.36
c81025_g1_i1	Protein nynrin-like	279.8 ± 28.8	1,876.9 ± 462.9	6.70
c50509_g1_i1	Ubiquitin-conjugating enzyme e2 r1-like	153.0 ± 41.6	1,020.9 ± 409.7	6.67
c64715_g1_i1	Protein phosphatase 1B-like	613.6 ± 367.0	2,661.5 ± 865.0	4.34
c78791_g3_i1	Apoptogenic protein mitochondrial-like	480.3 ± 402.1	1,849.8 ± 1,000.8	3.85
c88645_g4_i3	SRC kinase signaling inhibitor 1-like	1,280.5 ± 621.3	2,083.3 ± 136.7	1.63
c82175_g1_i2	Serine threonine-protein kinase 38-like	2,230.3 ± 898.8	3,210.7 ± 470.3	1.44
c86443_g1_i1	PAX3- and PAX7-binding protein 1	35,715.3 ± 6,540.5	37,981.7 ± 1,870.0	1.06
c92296_g2_i2	Tubulin alpha-1A chain-like	273,304.4 ± 3,334.7	285,511.9 ± 2,173.3	1.04
c13692_g1_i1	Ribosomal protein L29	45,614.5 ± 4,756.2	47,495.1 ± 1,940.2	1.04
c113887_g1_i1	Supervillin-like isoform x5	ND	1,359.3 ± 1,989.9	NA
c149_g1_i1	Star-related lipid transfer protein 13-like	ND	1,263.5 ± 1,570.3	NA
c57536_g1_i4	Neuronal pas domain-containing protein 2-like	ND	1,141.5 ± 500.8	NA
c79100_g1_i2	Chymotrypsin-like elastase family member 2a-like	1,171.3 ± 1,374.8	189.6 ± 126.0	-6.20
c92921_g2_i1	Period 1	1,780.3 ± 178.6	290.9 ± 229.9	-6.10
c57364_g1_i1	Pterin-4-alpha-carbinolamine dehydratase 2-like	1,400.4 ± 468.0	329.0 ± 91.2	-4.26
c91950_g1_i1	Period circadian protein homolog 2-like	2,653.6 ± 152.5	894.3 ± 80.5	-2.97
c85532_g1_i2	Immunoglobulin mu heavy chain	2,279.3 ± 909.3	814.9 ± 421.1	-2.80
c51266_g1_i1	Rho GTPase-activating protein 23-like isoform x9	6,700.8 ± 3,078.6	2,570.6 ± 1,353.0	-2.61
c55701_g1_i1	Synaptotagmin-c-like	1,958.7 ± 246.0	782.5 ± 395.3	-2.50
c76601_g1_i1	Period circadian protein homolog 1-like isoform x1	7,336.8 ± 672.7	3,601.1 ± 1,293.5	-2.04
c77744_g1_i1	Period circadian protein homolog 1-like isoform x1	4,053.6 ± 474.7	1,990.0 ± 376.7	-2.04

c85980_g1_i1	Nuclear receptor subfamily 1 group d member 2-like	3,015.9 ± 803.3	1,641.8 ± 347.3	-1.84
c96661_g4_i1	Polyadenylate-binding protein 2-like isoform x3	5,808.0 ± 822.8	3,673.1 ± 277.3	-1.58
c80106_g2_i1	Lipocalin precursor	66,744.1 ± 8,471.7	52,467.3 ± 4,148.2	-1.27
c77464_g1_i1	Protein FAM107B-like	9,727.2 ± 1,094.3	7,685.5 ± 863.4	-1.27
c32430_g1_i1	60S acidic ribosomal protein P2	54,877.1 ± 2,565.7	50,338.9 ± 3,830.0	-1.09
c140829_g1_i1	Unconventional myosin-xviiib-like	1,257.2 ± 1,929.3	ND	NA
c126916_g1_i1	Polycystin-1-like	1,127.9 ± 1,685.0	ND	NA

[a] RPKM: reads per kilobase of exon model per million mapped reads

[b] ND: not detected

[c] NA: not applicable

Figure legend

Fig. 1 Hierarchical clustering dendrograms from the RNA-seq analyzed by mapping to *Takifugu rubripes* genome (a) and contigs (b). The numbers represent independent samples. The vertical scale represents between-cluster distance