Tetrodotoxin functions as a stress relieving substance in juvenile tiger puffer *Takifugu rubripes*

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ABSTRACT

We tested whether tetrodotoxin (TTX) functions as a stress relieving substance in puffer fish. We orally administered TTX to the juveniles of hatchery-reared non-toxic tiger puffer *Takifugu rubripes* and measured the effects of TTX on brain corticotropin-releasing hormone (CRH) mRNA expression and plasma cortisol levels in comparison with effects in non-toxic juveniles. Firstly, the reciprocal connections of CRH and adrenocorticotropic hormone (ACTH) were confirmed by dual-label immunohistochemistry. CRH-immunoreactive (ir) cell bodies were detected in the hypothalamus and CRH-ir fibers were observed to project to ACTH-ir cells in the rostral pars distalis of the pituitary. Next, a TTX-containing diet (2.35 mouse units (517 ng)/g diet) or a non-toxic diet were fed to the fish for 28 days under a recirculating system. Standard length and body weight became significantly larger in the TTX-treated group. The degree of loss of the caudal fin, which is an indicator of the degree of agonistic interactions, where high values show a higher loss of caudal fin of a fish due to nipping by other individuals, was significantly lower in the TTX-treated group. Relative CRH mRNA expression levels in the brain and cortisol levels in the plasma were significantly lower in the TTX-treated group. These results indicate that TTX functions as a stress relieving substance by affecting the CRH-ACTH-cortisol axis and reducing agonistic interactions in tiger puffer juveniles.

Key words: TTX, CRH, ACTH, cortisol, stress, tiger puffer

1. Introduction

Tiger puffer *Takifugu rubripes* is well known to contain a potent neurotoxin "tetrodotoxin (TTX)", and is a commercially important fish species in Japan (Noguchi and Arakawa, 2008). Owing to the decrease in natural stocks of tiger puffer in Japan, artificial propagation is conducted widely both for aquaculture and stock enhancement programs (Katamachi and Ishida, 2013). These hatchery-reared tiger puffers are known to become non-toxic when fed non-toxic diets in an environment where TTX-bearing organisms are absent (Matsui et al., 1982; Noguchi et al., 2006; Saito et al., 1984). Further, agonistic interactions, such as nipping the fins and bodies of other conspecifics, are frequently observed in the hatcheries caused by stress from high individual densities (Ohgami and Suzuki, 1982; Han et al., 1994). Saito et al. (2002) found that the frequency of nipping behavior decreased in hatchery-reared juveniles of tiger puffer when they were fed with a TTX containing diet. When juvenile tiger puffer was fed with TTX containing diet for 10 days, the degree of loss of the caudal fin (DLCF) was lower in the orally TTX-administered fish than control fish (Sakakura et al., 2017), where DLCF is used as an indicator of degree of agonistic interactions in tiger puffer where high values show higher loss of caudal fin of a fish due to nipping by other individuals (Shimizu et al., 2006). Assuming that nipping is caused by rearing stress, these findings suggest that TTX functions as a stress relieving substance in juvenile tiger puffer.

Stress is regulated by the hypothalamo-pituitary-interrenal (HPI) axis in fish. Stress stimulates the synthesis of corticotropin-releasing hormone (CRH) in the hypothalamus, which in turn stimulates the synthesis of the proopiomelanocortin (POMC) and cleavage of POMC to adrenocorticotropic hormone (ACTH) in the pituitary (see Pankhurst, 2011). ACTH stimulates the release of cortisol from the interrenal gland and then cortisol increases glucose through glucogenesis. In teleost fish, CRH-immunoreactive (ir) cell bodies in the nucleus preopticus project directly to ACTH cells in the rostral pars distalis (RPD) and to α-melanocyte stimulating hormone (α-MSH) cells in the pars intermedia (PI) of the pituitary (see Flik et al., 2006).

Recently, the presence of TTX in the brain of the tiger puffer has been reported. TTX was detected by immunohistochemistry in the brain of wild toxic tiger puffer and orally TTX-administered hatchery-reared tiger puffer (Okita et al., 2013). Moreover, TTX was detected by LC/MSMS analysis in the brain of orally TTX-administered hatchery-reared tiger puffer (Sakakura et al., 2017). Considering that TTX is present in the brain of toxic tiger puffer and that the brain (hypothalamus) is the center of endocrine system, we hypothesized that TTX in the brain relieves rearing stress by affecting the CRH-ACTH-cortisol axis. In our previous study, the oral administration of TTX to hatchery-reared non-toxic tiger puffer juveniles resulted in the accumulation of the toxin in various tissues, such as the skin, muscle, liver, and brain, similar to that seen in wild toxic juveniles (Sakakura et al., 2017). This indicates that fish with orally administered TTX can be considered to reflect the physiological characteristics of wild toxic fish.

Therefore, in the present study, we first confirmed the reciprocal connection of CRH and ACTH in the pituitary of juvenile tiger puffer by dual-label immunohistochemistry. Then, we examined the effects of oral administration of TTX on somatic growth and agonistic interactions, brain CRH mRNA expression, and plasma cortisol and glucose levels in the hatchery-reared non-toxic juvenile tiger puffer in order to examine our hypothesis that TTX functions as a stress relieving substance by affecting the CRH-ACTH-cortisol axis.

2. Materials and methods

2.1. Dual-label immunohistochemistry for CRH and ACTH

The juveniles of hatchery-reared non-toxic tiger puffer (mean body weight (BW): 1.3 g) obtained from Nagasaki Fishery Public Corporation, Sasebo, Nagasaki, Japan, were anesthetized by immersion in 200 ppm of MS222. The brain with pituitary was excised, fixed with Bouin's fluid for 24 hours at 4 ^{o}C, rinsed in cold 70% ethanol, dehydrated through a graded series of ethanol concentrations, and embedded in paraplast (Monoject, Sherwood Medical, St Louis, MO, USA). Sagittal sections were cut at 8 μm and mounted on MAS-GP coated slides (Matsunami, Osaka, Japan). To examine the innervation of CRH-ir fibers to ACTH cells in the pituitary, dual-label immunohistochemistry was conducted according to Amano et al (2016), using a rabbit polyclonal antibody raised against human/mouse/rat CRH (Cat. # AB-02, Advanced Targeting Systems, San Diego, CA, USA) and the mouse monoclonal antibody raised against ACTH (Cat. # MS-452-P0, Thermo Scientific, Fremont, CA, USA). CRH and ACTH immunoreactivities were visualized by 3,3'-diaminobenzidine tetrahydrochloride (DAB, brown) and nitro blue tetrazolium chloride, and 5-bromo-4-chloro-3-indolyl phosphate, toluidine salt (NBT/BCIP, blue), respectively. mRNA sequences encoding CRH in tiger puffer has been updated in NCBI database (NCBI Reference Sequence: XM_003967938.1). The deduced amino acid sequence of tiger puffer CRH is SEDPPISLDLTFHLLREMMEMSKAEQLAQQAQNNRIMMELV-NH_2 and the sequence identity with human/mouse/rat CRH is 73%. The cross-reactivity of the anti-CRH antibody against CRH family peptides such as urocortin-I, II, III, urotensin-I, and sauvagine, was less than 0.01%, indicating the specificity of the antibody (Amano et al., 2016). To test the specificity of the immunohistochemical reactions for CRH, control sections were incubated in antisera that had been pre-absorbed overnight at 4 ^{o}C with an excess amount of synthetic CRH (2.5 μg CRH in 1 mL of diluted antiserum). The subsequent procedure was identical to that used for the experimental sections.

2.2. TTX administration experiment

2.2.1. Preparation of the TTX containing diet

The present study aimed to investigate the effects of dietary TTX on the growth performance and stress-related hormone levels in juvenile tiger puffer. Fish meal is a common ingredient for fish diets, however, it contains various nutritional factors that may affect the physiology of fish. Thus, we used casein-based semi-purified diets (1.2 mm in diameter) with small amount of fish meal in this study following the method described by Matsunari et al. (2008) as shown in Table 1. TTX (Wako Pure Chemical, Osaka, Japan) was dissolved in Milli-Q water (Merck Millipore, Billerica, MA, USA) at a toxicity of 46 mouse units (MU)/mL. TTX solution (32.5 mL) and 7.5 g of soy lecithin (Nacalai Tesque Inc., Kyoto, Japan) were homogenized in an ice bath for 3 min at 14,000 rpm. Then, a TTX containing emulsion was made by adding 20 mL of salad oil and homogenizing the TTX solution in an ice bath for 3 min at 14,000 rpm. The control emulsion was also prepared in the same manner as the TTX containing emulsion but replacing the amount of TTX solution with Milli-Q water. Each emulsion was sprayed onto 250 g of diet material, respectively. Concentrations of adsorbed TTX in the diet were measured in diet samples. TTX were extracted with 0.1% acetic acid following the standard protocol by Japan Food Hygiene Association (2015). Then, the extract was partially purified with Bio-Gel P-2 column (Bio-Rad Laboratories Inc., Hercules, CA, USA) and the absorbed TTX was eluted with 0.05 M AcOH from the gel. The TTX fraction was analyzed by LC/MS/MS according to the method described by Nakashima et al (2004) and Gao et al (2019). The chromatography was carried out on an Alliance 2690 Separations Module (Waters, Milford, MA, USA) with a Mightysil RP-18 GP column (2.0 x 250 mm, Kanto Chemical Co., Inc., Japan). The mobile phase comprised 30 mM heptafluorobutyric acid in 1 mM ammonium acetate buffer (pH 5.0), at a flow rate of 0.2 ml/min. The eluate was introduced into a Quattro micro^{TM} API

detector (Waters), with a desolvation temperature of 350 °C, source block temperature of 120 °C, and cone voltage of 50 V. Therein, the TTX was ionized by positive-mode electrospray ionization and then monitored as product ions (collision voltage 38 V) at *m*/*z* 162 (for quantitative measure) and 302 (for qualitative measure), and as the precursor ion at *m*/*z* 320, using a MassLynx^{TM} NT operating system (Waters). The amount of TTX (in ng) determined by LC/MS/MS was converted to MU based on the specific toxicity of TTX (220 ng/MU). The effective concentrations of TTX in the diet was 2.35 MU (517 ng)/g·diet.

2.2.2. Experimental fish

Hatchery-reared non-toxic tiger puffer juveniles (mean BW: 1.7 g) were obtained from Nagasaki Fishery Public Corporation. Fish were transferred to The Graduate School of Fisheries and Environmental Sciences, Nagasaki University on June 7, 2017. The fish were kept as a stock in a 120-L cylindrical tank with pure-oxygen supply in a temperature-controlled room at 25°C. The experiment was performed following the guidelines of the animal care committee of Nagasaki University and Kitasato University.

On June 8, 2017 (Day 0), a total of 40 fish were taken from the stock tank and were randomly divided into two groups. Each fish was anesthetized using 200 ppm of MS222, their total length (TL) and standard length (SL) were measured by a digital caliper (CD20-GM; Mitsutoyo Cooperation, Kanagawa, Japan), and BW was weighed with an electric balance (PB153-S; Mettler-Toledo, OH, USA) with an accuracy of up to two decimal points. Then the fish was marked individually using visible implant elastomer tags (VIE; Northwest Marine Technology, WA, USA) at the base of the anal fin according to Shimizu et al (2008) to track individual growth performance. Fish were kept in 200-L black polyethylene tank (20 fish each) equipped with recirculating system (about 50 L/h) and were fed a non-toxic commercial diet (Otohime S2; Marubeni Nisshin Feed, Tokyo, Japan) at satiety at 9:00 and 15:00 for 7 days for acclimatization to the experimental settings. Then, fish were fed the non-toxic test diet at satiety at 9:00 and 15:00 until June 21, 2017 (Day 13) to acclimatize to test diets. The TTX-containing diet and the non-toxic test diet were fed to the TTX-treated group and control group, respectively, for 28 days, from June 22, 2017 (Day 14) to July 19, 2017 (Day 41).

2.2.3. Fish sampling

Fish were sampled at Day 42, on July 20, 2017. All fish that had survived were anesthetized using 200 ppm of MS222, and the TL, SL, and BW were measured; 17 and 18 fish survived in the control and the TTX-treated groups, respectively.

DLCF was calculated with following equation:

DLCF (%) = 100× {1–(L*th*-L*sh*)/ (L*tw*-L*sh*)}.

Where L*th* and L*sh* indicate the TL and SL of a measured fish, and L*tw* is an estimated TL from the wild fish of the same SL that has no loss of caudal fin from the following equation:

L*tw*=1.1806×L*sh*+6.0142 (Shimizu et al. 2006).

Specific growth rate (SGR) was also calculated as follows:

SGR (BW/day) = {ln (final BW)–ln (initial BW)} ×100/day.

For measurements of the brain CRH mRNA expression and plasma cortisol and glucose levels, 12 fish were randomly selected from both groups. Blood was collected from the sinus venosus using heparinized syringe to measure the plasma levels of cortisol and glucose. Blood samples were centrifuged at 2500 *g* for 15 min and plasma was stored at –35 ºC until analysis.

To measure brain CRH mRNA levels by quantitative Real-Time PCR (qRT-PCR), the brain without the pituitary was quickly dissected out, immersed in RNAlater (Sigma-Aldrich, CA, USA), and stored at –80 ºC until analysis.

To confirm the accumulation of TTX in the fish, liver, skin, and muscle of each fish were dissected and were stored at –20 ºC until LC/MS/MS analysis (Nakashima et al., 2004; Gao et al.,

2019). TTX content in each tissue was pooled for each individual to calculate the TTX amount per BW.

2.2.4. Quantitative Real-Time PCR for CRH

Total RNA was prepared from each brain tissue sample using the RNeasy Lipid Tissue Mini Kit (Qiagen, Germantown, MD, USA) and treated with the RNase-Free DNase Set (Qiagen) to eliminate genomic DNA contamination. The RNA yield was measured spectrophotometrically by absorbance at 260 nm. Single-strand cDNA was reverse transcribed from 1 μg of total RNA using the PrimeScript™ 1st strand cDNA Synthesis Kit (Takara-Bio, Shiga, Japan). All procedures were performed according to the manufacturer's instructions.

qRT-PCR was performed with specific primers and TaqMan Minor Groove Binder (MGB) probes designed from sequence data of tiger puffer from GenBank; CRH (GenBank accession number; XM_003967938.1) and β-actin (GenBank accession number; XM_003964421.1). All primer pairs and hybridization probes were designed using qPCR Primer & Probe Design Tool (Eurofins Genomics, Ebersberg, Germany), as shown in Supplementary Fig. S1.

qRT-PCR was conducted using StepOnePlus™ Real Time PCR System (Applied Biosystems, CA, USA). We used TaqMan® One Step PCR Master Mix Reagents Kit (Applied Biosystems). Each well contained a reaction mixture of 5 μL of 2× Master Mix without UNG, 0.4 μL of forward primer (10 μM), 0.4 μL of reverse primer (10 μM), 0.16 μL of TaqMan MGB probe (10 μM), 0.25 μL of 40× MultiScribe™ and RNase Inhibitor Mix, 1.79 μL of sterilized distilled water, and 2 μL of first-strand cDNA sample. The cycling parameters were as follows: 10 min at 95 ºC followed by 50 cycles of 95 ºC for 30 sec and 60 ºC for 2 min. Ct (threshold cycle) values corresponding to the cycle number at which the fluorescent emission was monitored in real time were measured. The threshold and Ct values acquired via qRT-PCR were used to analyze CRH mRNA levels according to the $2^{-\Delta\Delta Ct}$ method. Final output was expressed as relative CRH mRNA expression by correcting values of corresponding β-actin. To validate this qRT-PCR for CRH, the amplification efficiencies (e) of CRH and β-actin were examined by calculating $e = 10^{-1/slope} - 1$. Each sample was analyzed in triplicate.

2.2.5. Plasma cortisol levels

Plasma cortisol levels were measured by a time-resolved fluoroimmunoassay (TR-FIA) for cortisol (Yamada et al., 2002). Cross-reactivities of the anti-cortisol antibody (Cat. # FKA-402, Cosmo-Bio, Tokyo, Japan) against chemically resembled steroids are as follows: deoxycorticosterone (12%), 18-OH-deoxycorticosterone (8.5%), corticosterone (8%), 17α-OH-progesterone (5%), progesterone (2%), aldosterone (0.5%), androstendione (0.4%), testosterone (0.1%), dehydroeplandrosterone (less than 0.01%), and estradiol (less than 0.01%) (Amano et al., 2016).

2.2.6. Plasma glucose levels

Plasma glucose levels were measured by Autokit Glucose (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), according to the manufacturer's instructions.

2.2.7. Statistics

Fisher's exact test was performed to compare the survival rate at Day 42 of treatment. All collected data from each treatment group were tested the same day for normality by Shapiro-Wilk normality test and for equal variance by the Bartlett test. When data were recognized as parametric values, then the Student's *t*-test was performed to compare the difference between treatments (SGR and glucose). Wilcoxon rank sum test was performed between treatments in case of non-parametric values (CRH and cortisol levels). Growth parameters (SL, BW) and parameters for agonistic interactions (DLCF) were judged as non-parametric values. Then, differences in values of SL, BW

and DLCF between treatment groups during the experimental period were compared using two-way repeated ANOVA of Aligned Rank Transformed Data followed by pairwise comparison of least squares means with Bonferroni adjustment.

Statistical analysis was carried out using R. version 3.5.2 (R: A language and environment for statistical computing, R Foundation for Statistical Computing, Vienna, Austria, http://www.R-project.org/ "Accessed 2 April 2019") with 'ARTool' and 'emmeans' packages, and p-values < 0.05 were considered significant in all analyses.

3. Results

3.1. Dual-label immunohistochemistry for CRH and ACTH

CRH-ir cell bodies were detected in the hypothalamus and CRH-ir fibers were observed to project to ACTH-ir cells in the RPD of the pituitary (Fig. 1A, B, D). No CRH-ir cell bodies or fibers were observed when the anti-CRH antibody was pre-absorbed overnight at 4 ^{o}C with an excess amount of synthetic human/mouse/rat CRH (Fig. 1C), indicating the specificity of immunoreaction. ACTH-ir cells, and α-MSH-ir cells cross-reacted with anti-ACTH antibody, were detected in the RPD and the PI of the pituitary, respectively (Fig. 1D). CRH-ir fibers were observed to project to ACTH-ir cells in the RPD of the pituitary (Fig. 1E). CRH-ir fibers were also observed to project to α-MSH-ir cells in the PI of the pituitary (Fig. 1D).

3.2. TTX administration experiment

3.2.1. Survival and growth of fish

Survival rate of the control (85%) and the TTX-treated groups (90%) was not significantly different (Fisher's exact test, p=1.0). Fish fed with the TTX-containing diet showed significantly larger SL and BW than those fed with the control diet (Aligned Rank Transform for nonparametric factorial ANOVAs, factors=diet×day, df=1, F=9.1848, p<0.01 for SL, and F=27.785, p<0.001 for BW) (Fig. 2A, B). SGR of the TTX-treated group (4.3 ± 0.5, mean ± SD, n=18) was also significantly higher than that of the control group (3.8 ± 0.5, n=17; t-test, t = –2.7735, df=31.716, p<0.01), indicating that juveniles of the TTX-treated group showed better growth than those of the control group.

3.2.2. Accumulation of TTX

TTX was detected in all the fish of the TTX-treated group (0.4 ± 0.2 MU (88 ± 44 ng)/g BW, n=18), whereas TTX was below detectable limit (<0.05 MU (11 ng)/ml sample) in the fish of the control group.

3.2.3. DLCF (%)

On the initial sampling (Day 0), no significant differences were observed in DLCF between the groups. On the final sampling (Day 42), DLCF was significantly smaller in the TTX-treated group than that in the control group (Aligned Rank Transform for nonparametric factorial ANOVAs, factors=diet×day, df=1, F=5.5398, p= 0.025) (Fig. 2C).

3.2.4. Relative CRH mRNA expression levels in the brain

The amplification efficiencies of the qRT-PCR for CRH and β-actin were 1.026 and 0.972, respectively, and both standard curves were regarded as parallel (Supplementary Fig. S2), indicating the validity of this qRT-PCR. Relative CRH mRNA expression levels in the brain were significantly lower in the TTX-treated group than those in the control group (Wilcoxon-test, W=116, p=0.012) (Fig. 3).

3.2.5. Plasma cortisol and glucose levels

Plasma cortisol levels were significantly lower in the TTX-treated group than those in the control group (Wilcoxon-test, W=116, p=0.010) (Fig. 4A). As for plasma glucose levels, no significant differences were observed between the groups (t-test, t = 0.11859, df=21.999, p=0.9067) (Fig. 4B).

4. Discussion

In the present study, the reciprocal connections of CRH and ACTH in the hatchery-reared non-toxic juvenile tiger puffer were first demonstrated by dual-label immunohistochemistry. Oral administration of TTX to hatchery-reared non-toxic juvenile tiger puffer resulted in lower brain CRH mRNA expression and plasma cortisol level when compared with the control fish. Moreover, fish fed with the TTX-containing diet showed lower caudal fin loss, indicating less agonistic interactions such as nipping among the TTX-treated fish, which is the same as in the previous studies (Saito et al., 2002; Sakakura et al., 2017). These evidences support our hypothesis that TTX functions as a stress relieving substance. Thus, our results propose a novel physiological function of TTX in puffer fish, as described below.

Okita et al. (2013) examined the immunohistochemical localization of TTX in the brain of a TTX-administered juvenile tiger puffer and detected a high TTX concentration at the molecular layer and in Purkinje cells in the brain. It is known that Purkinje cells serve as the sole output of the cerebellar cortex of the cerebellum (Voogd and Glickstein, 1998). Considering that the teleost cerebellar corpus may play a role in motor learning and motor control, it is indicated that TTX transferred to the brain is neurologically functional in juvenile tiger puffer. Incidentally, the brain (hypothalamus) is the center of endocrine system; endocrine system of the vertebrate is regulated by the hypothalamo-pituitary-target organ axis. Our present results indicate that TTX transferred to the brain is neuro-endocrinologically functional in juvenile tiger puffer, because oral administration of TTX affected the gene expression of one of the hypothalamic hormones (neuropeptides), CRH.

As for plasma glucose levels, no significant differences were observed between the groups, although plasma cortisol levels were significantly lower in the TTX-treated group. In general, when fish are subjected to stress, energy metabolism increases to cope with stress response, and glucose is used as the main energy resources (Wendelaar Bonga, 1997; Fabbri et al., 1998). Thus, it is speculated that prolonged rearing stress of a total of 42 days in the present study, resulted in a sustained consumption of energy resources especially in the control group, as has been reported in rainbow trout *Onchorhynchus mykiss* (Conde-Sieira et al., 2014).

Oral administration of TTX on hatchery-reared non-toxic juvenile tiger puffer stimulated somatic growth in the present study. Since we used casein-based semi-purified diets to exclude various nutritional factors that may stimulate food intake of the tiger puffer, the orexigenic effect of TTX is considered to be detected. Here, a question arises how TTX stimulates food intake. One possible explanation is that TTX reduces CRH gene expression in the brain. It has been demonstrated that CRH suppresses appetite and feeding behavior in the goldfish *Carassius auratus* (Bernier and Peter, 2001; Bernier and Craig, 2005; Bernier, 2006; Maruyama et al., 2006; Matsuda et al., 2013). Supposing that this is also true for the tiger puffer and considering that oral administration of TTX resulted in lower brain CRH mRNA expression, it is suggested that decreased brain CRH mRNA in the TTX-treated group consequently stimulated food intake compared to the control group. Furthermore, it has also been reported that CRH increases locomotor activity in Chinook salmon *O. tshawytscha* (Clements et al., 2002; Lowry and Moore, 2006) and rainbow trout (Carpenter et al., 2007). If this is also true for the tiger puffer, decreased brain CRH mRNA in the TTX-treated group may have inhibited locomotor activity compared to the control group, resulting in reduction of energy loss. More precise research integrating the mode of action of TTX in the brain and behavioral differences caused by TTX administration in puffer fish is needed to clarify this hypothesis.

It is widely accepted that puffer fish do not produce TTX by themselves. Puffer fish accumulate TTX by ingesting toxic food organisms. Indeed, hatchery-reared tiger puffer is known to become non-toxic when fed with non-toxic diets in an environment where TTX-bearing organisms are absent (Matsui et al., 1982; Noguchi et al., 2006; Saito et al., 1984). Moreover, it has been known that TTX levels in the wild tiger puffer juveniles vary largely due to the location the fish are

collected in different years (0.6-6.0 MU/fish, Shimizu et al. 2007; Sakakura et al. 2017). Thus, it has been regarded that TTX in puffer fish is not indispensable for maintenance of life; then, a question arises why puffer fish possess TTX. One convincing hypothesis is that TTX is involved in avoidance from predators (Itoi et al., 2014). Indeed, TTX is primary localized in the larval body surface of the tiger puffer, revealed by immunohistochemistry, and when predators ingested the puffer fish larva (0-4 days post-hatch), they quickly spat out the larva (Itoi et al., 2014). Many predatory fish seem to quickly sense TTX on the body surface of the prey larvae; for example, it has been reported through electrophysiological method that rainbow trout and arctic char *Salvelinus alpinus* can sense extremely low levels of TTX (Yamamori et al., 1988). The reason why toxic wild tiger puffer juveniles possess TTX in the brain could also be related to the fear response. With regard to the fear response, it has been reported that a difference exists between non-toxic hatchery-reared tiger puffer juveniles and toxic wild juveniles (Shimizu et al., 2007, 2008); when tiger puffer juveniles are moved to a new environment, wild juveniles swim around the bottom, whereas non-toxic hatchery-reared juveniles swim at the water surface. It has been shown that behavioral deficits in the fear response can be a major cause of mortality in hatchery-reared juveniles shortly after their release (Shimizu et al., 2007, 2008). The reason for higher survival rate of toxic wild fish in these studies may be not only accumulated TTX in the skin of fish, which acts as a predator defense chemical, but also because TTX in the brain activates the expression of the fear response, which is advantageous for survival. Further study is needed to clarify whether oral TTX administration affects the fear response in non-toxic hatchery-reared tiger puffer juveniles.

In summary, we have indicated that oral administration of TTX reduces rearing stress by affecting the CRH-ACTH-cortisol axis in the juvenile tiger puffer. The relationship between stress and CRH activity of the tiger puffer should be clarified in future studies.

5. Conclusions

To investigate a physiological function of TTX in puffer fishes, we tested a hypothesis whether TTX functions as a stress relieving substance. Our results indicate that TTX functions as a stress relieving substance by affecting the CRH-ACTH-cortisol axis and reducing agonistic interactions in tiger puffer juveniles.

Ethical statement

The authors declare that this manuscript complies with the Elsevier Ethical Guidelines for Journal Publication.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

References

Amano, M., Amiya, N., Yokoyama, T., Onikubo, K., Yamamoto, N., Takahashi, A., 2016. Immunohistochemical detection of corticotropin-releasing hormone (CRH) in the brain and pituitary of the hagfish, *Eptatretus burgeri*. Gen. Comp. Endocrinol. 236, 174–180. https://doi.org/10.1016/j.ygcen.2016.07.018.

Bernier, N.J., Peter, R.E., 2001. The hypothalamic–pituitary–interrenal axis and the control of food intake in teleost fish. Comp. Biochem. Physiol. 129B, 639–644. https://doi.org/10.1016/S1096-4959(01)00360-8.

Bernier, N.J., Craig, P.M., 2005. CRF-related peptides contribute to stress response and regulation of appetite in hypoxic rainbow trout. Am. J. Physiol. Regul. Integr. Comp. Physiol. 289, R982–R990. https://doi.org/10.1152/ajpregu.00668.2004.

Bernier, N.J., 2006. The corticotrophin-releasing factor system as a mediator of the appetite-suppressing effects of stress in fish. Gen. Comp. Endocrinol. 146, 45–55. https://doi.org/10.1016/j.ygcen.2005.11.016.

Carpenter, R.E., Watt, M.J., Forster, G. L., Øverli, Ø., Bockholt, C., Renner, K.J., Summers, C.H., 2007. Corticotropin releasing factor induces anxiogenic locomotion in trout and alters serotonergic and dopaminergic activity. Horm. Behav. 52, 600–611. https://doi.org/10.1016/j.yhbeh.2007.07.012.

Clements, S., Schreck, C.B., Larsen, D.A., Dickhoff, W.W., 2002. Central administration of corticotropin-releasing hormone stimulates locomotor activity in juvenile chinook salmon (*Oncorhynchus tshawytscha*). Gen. Comp. Endocrinol. 125, 319–327. https://doi.org/10.1006/gcen.2001.7707.

Conde-Sieira, M., Muñoz, J.L., López-Patiño, M.A., Gesto, M., Soengas, J.L., Míguez, J.M., 2014. Oral administration of melatonin counteracts several of the effects of chronic stress in rainbow trout. Domest. Anim. Endocrinol. 46, 26–36. https://doi.org/10.1016/j.domaniend.2013.10.001.

Fabbri, E., Capuzzo, A., Moon, T.W., 1998. The role of circulating catecholamines in the regulation of fish metabolism: an overview. Comp. Biochem. Physiol. C. 120, 177–192. https://doi.org/10.1016/S0742-8413(98)10017-8.

Flik, G., Klaren, P.H.M., Van den Burg, E.H., Metz, J.R., Huising, M.O., 2006. CRF and stress in fish. Gen. Comp. Endocrinol. 146, 36–44. https://doi.org/10.1016/j.ygcen.2005.11.005.

Gao, W., Kanahara, Y., Yamada, M., Tatsuno, R., Yoshikawa, H., Doi, H., Takatani, T., Arakawa, O., 2019. Contrasting toxin selectivity between the marine pufferfish *Takifugu pardalis* and the freshwater *pufferfish Pao suvattii*. Toxins. 11, 470. doi:10.3390/toxins11080470.

Han, K., Matsui, S., Furuichi, M., Kitajima, C., 1994. Effect of stocking density on growth, survival rate, and damage of caudal fin in larval to young puffer fish, *Takifugu rubripes*. SUISANZOSHOKU. 42, 507–514. https://doi.org/10.11233/aquaculturesci1953.42.507.

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. https://doi.org/10.1016/j.toxicon.2013.11.003.

Japan Food Hygiene Association (2015) Puffer toxin. In: Environmental Health Bureau, Ministry of Health and Welfare (ed.), Shokuhin Eisei Kensa Shishin (Manual for Methods for Food Sanitation Testing). Tokyo, pp. 813–820 (in Japanese)

Katamachi, D., Ishida, M., 2013. Stock assessment and evaluation for tiger puffer in the Sea of Japan, the East China Sea and the Seto Inland Sea (fiscal year 2012). In: Marine fisheries stock assessment and evaluation for Japanese waters (fiscal year 2012/2013). Fisheries Agency and Fisheries Research Agency of Japan, Tokyo, pp 1589–1613 (in Japanese).

Lowry, C.A., Moore, F.L., 2006. Regulation of behavioural responses by corticotrophin-releasing

442 factor. Gen. Comp. Endocrinol. 146, 19–27. https://doi.org/10.1016/j.ygcen.2005.12.006.
443 Maruyama, K., Miura, T., Uchiyama, M., Shioda, S., Matsuda, K., 2006. Relationship between
444 anorexigenic action of pituitary adenylate cyclase-activating polypeptide (PACAP) and that of
445 corticotropin-releasing hormone (CRH) in the goldfish, *Carassius auratus*. Peptides. 27,
446 1820–1826. https://doi.org/10.1016/j.peptides.2006.01.013.
447 Matsuda, K., Hagiwara, Y., Shibata, H., Wada, K., 2013. Ovine corticotropin-releasing hormone
448 (CRH) exerts an anxiogenic-like action in the goldfish, *Carassius auratus*. Gen. Comp.
449 Endocrinol. 188, 118–122. https://doi.org/10.1016/j.ygcen.2013.01.001.
450 Matsui, T., Sato, H., Hamada, S., Shimizu, C., 1982. Comparison of toxicity of the cultured and
451 wild puffer fish *Fugu niphobles*. Bull. Jpn. Soc. Sci. Fish. 48, 253.
452 https://doi.org/10.2331/suisan.48.253.
453 Matsunari, H., Furuita, H., Yamamoto, T., Kim, S.K., Sakakura, Y., Takeuchi, T., 2008. Effect of
454 dietary taurine and cystine on growth performance of juvenile red sea bream *Pagrus major*.
455 Aquaculture. 274, 142–147. https://doi.org/10.1016/j.aquaculture.2007.11.002.
456 Nakashima, K., Arakawa, O., Taniyama, S., Nonaka, M., Takatani, T., Yamamori, K., Fuchi, Y.,
457 Noguchi, T., 2004. Occurrence of saxitoxins as a major toxin in the ovary of a marine puffer
458 *Arothron firmamentum*. Toxicon. 43, 207–212. https://doi.org/10.1016/j.toxicon.2003.05.001.
459 Noguchi, T., Arakawa, O., 2008. Tetrodotoxin – distribution and accumulation in aquatic organisms,
460 and cases of human intoxication. Mar. Drugs. 6, 220–242. https://doi.org/10.3390/md20080011.
461 Noguchi, T., Arakawa, O., Takatani, T., 2006. Toxicity of puffer fish *Takifugu rubripes* cultured in
462 netcages at the sea or aquaria on land. Comp. Biochem. Physiol. D1, 153–157.
463 https://doi.org/10.1016/j.cbd.2005.11.003.
464 Ohgami, H., Suzuki, Y., 1982. The influence of rearing conditions on survival and cannibalism on
465 fingerlings of tiger puffer (*Takifugu rubripes* T. et S.). Bull. Shizuoka Prefect. Fish. Exp. Stn.
466 16, 79–85. (in Japanese)
467 Okita, K., Takatani, T., Nakayasu, J., Yamazaki, H., Sakiyama, K., Ikeda, K., Arakawa, O.,
468 Sakakura, Y., 2013. Comparison of the localization of tetrodotoxin between wild pufferfish
469 *Takifugu rubripes* juveniles and hatchery-reared juveniles with tetrodotoxin administration.
470 Toxicon. 71, 128–133. https://doi.org/10.1016/j.toxicon.2013.05.018.
471 Pankhurst, N.W., 2011. The endocrinology of stress in fish: an environmental perspective. Gen.
472 Comp. Endocrinol. 170, 265–275. https://doi.org/10.1016/j.ygcen.2010.07.017.
473 Saito, T., Horiguchi, S., Saito, H., Maita, T., 2002. Effect of tetrodotoxin feeding on bite behavior of
474 cultured tiger puffer (*Takijugu rubripes*). J. Sch. Mar. Sci. Technol. Tokai Univ. 55, 79–87. (in
475 Japanese with English abstract)
476 Saito, T., Maruyama, J., Kanoh, S., Jeon, J.K., Noguchi, T., Harada, T., Murata, O., Hashimoto, K.,
477 1984. Toxicity of the cultured puffer fish Fugu *rubripes rubripes* along with their resistibility
478 against tetrodotoxin. Bull. Jpn. Soc. Sci. Fish. 50, 1573–1575. (in Japanese with English
479 abstract) https://doi.org/10.2331/suisan.50.1573.
480 Sakakura, Y., Takatani, T., Nakayasu, J., Yamazaki, H., Sakiyama, K., 2017. Administration of
481 tetrodotoxin protects artificially raised juvenile tiger puffer *Takifugu rubripes* from predators.
482 Fish. Sci. 83, 191–197. https://doi.org/10.1007/s12562-016-1046-0.
483 Shimizu, D., Sakiyama, K., Takahashi, Y., 2006. Predation of stocked hatchery-reared juveniles of
484 ocellate puffer *Takifugu rubripes* in salt pond mesocosm. Nippon Suisan Gakkaishi. 72,
485 886–893. (in Japanese with English abstract) https://doi.org/10.2331/suisan.72.886.
486 Shimizu, D., Sakiyama, K., Sakakura, Y., Takatani, T., Takahashi, Y., 2007. Predation differences
487 between wild and hatchery-reared tiger puffer, *Takifugu rubripes*, juveniles in a salt pond
488 mesocosm. Nippon Suisan Gakkaishi. 73, 461–469. (in Japanese with English abstract)
489 https://doi.org/10.2331/suisan.73.461.
490 Shimizu, D., Sakiyama, K., Sakakura, Y., Takatani, T., Takahashi, Y., 2008. Quantitative evaluation
491 of post-release mortality using salt pond mesocosm: case studies of hatchery and wild juvenile

tiger puffer. Rev. Fish. Sci. 16, 195–203. https://doi.org/10.1080/10641260701681755.
Voogd, J., Glickstein, M., 1998. The anatomy of the cerebellum. Trends. Cogn. Sci. 2, 307–313. https://doi.org/10.1016/S1364-6613(98)01210-8.
Wendelaar Bonga, S.E., 1997. The stress response in fish. Physiol. Rev. 77, 591–625. https://doi.org/10.1152/physrev.1997.77.3.591.
Yamada, H., Satoh, R., Fujimoto, Y., Takaji, K., Hakuba, T., Chiba, H., Iwata, M., 2002. Circadian changes in serum concentrations of steroids in Japanese char *Salvelinus leucomaenis* at the stage of final maturation. Zool. Sci. 19, 891–898. https://doi.org/10.2108/zsj.19.891.
Yamamori, K., Nakamura, M., Matsui, T., Hara, T.J., 1988. Gustatory responses to tetrodotoxin and saxitoxin in fish: a possible mechanism for avoiding marine toxins. Can. J. Fish. Aqua. Sci. 45, 2182–2186. https://doi.org/10.1139/f88-253.

Highlights

We tested whether TTX functions as a stress relieving substance in *Takifugu rubripes*.

CRH-ir fibers were observed to project to ACTH-ir cells in the pituitary.

The degree of loss of the caudal fin was lower in the TTX-treated group.

CRH mRNA levels and cortisol levels were lower in the TTX-treated group.

TTX affects the CRH-ACTH-cortisol axis and reduces agonistic interactions.

Table 1. Composition of the experimental diet

Ingredients (% dry weight)	Control	TTX
Casein	51.7	51.7
Fish meal	10.0	10.0
Krill meal	5.0	5.0
Soybean lectin	14.0	14.0
α-Starch	5.0	5.0
Feed oil	4.5	4.5
Others (vitamin mix, etc.)	9.8	9.8
Tetrodotoxin	0.0	2.35 MU

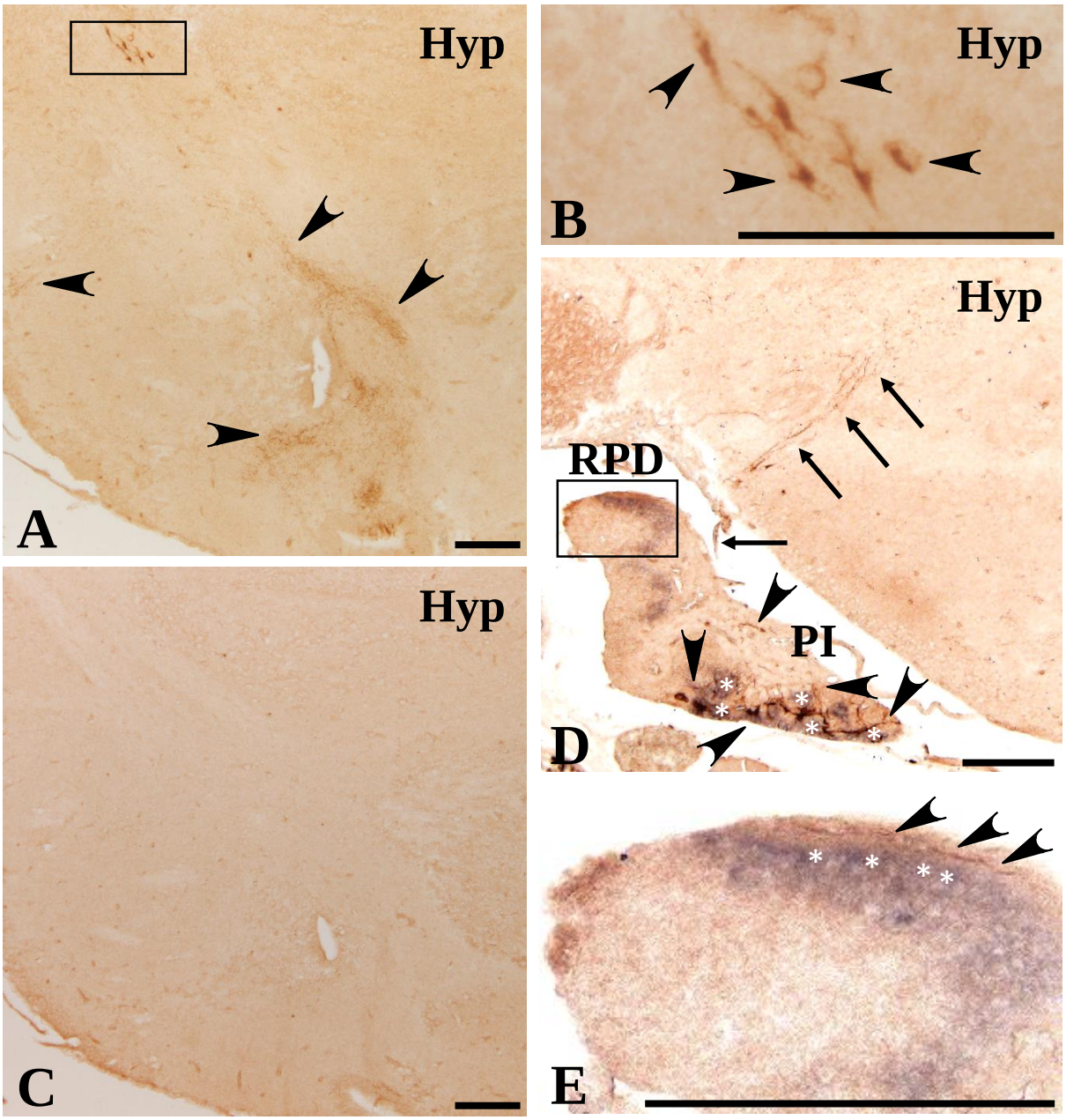

Fig. 1. (A) Sagittal section through the hypothalamus. CRH-ir cell bodies (boxed area) and fibers (brown, arrowheads) are observed. (B) Higher magnification of boxed area in 'A'. CRH-ir cell bodies (brown, arrowheads) are observed. (C) Adjacent section of 'A'. No CRH-ir cell bodies and fibers are observed when the anti-CRH antibody was pre-absorbed overnight at 4 ^{o}C with an excess amount of synthetic human/mouse/rat CRH. (D) Sagittal section through the hypothalamus and the pituitary. CRH-ir fibers projecting to the pituitary are found (brown, arrows). ACTH-ir cells in the RPD (boxed area) and α-MSH-ir cells in the PI of the pituitary (white asterisks) are observed. CRH-ir fibers (brown, arrowheads) are in close apposition with α-MSH-ir cells (blue, white asterisks) in the PI of the pituitary. (E) Higher magnification of the boxed area in 'D'. CRH-ir fibers (brown, arrowheads) are in close apposition with ACTH-ir cells (blue, white asterisks) in the RPD of the pituitary. Left indicates the rostral. Bars indicate 100 μm. Hyp hypothalamus, PI pars intermedia of the pituitary, RPD rostral pars distalis of the pituitary.

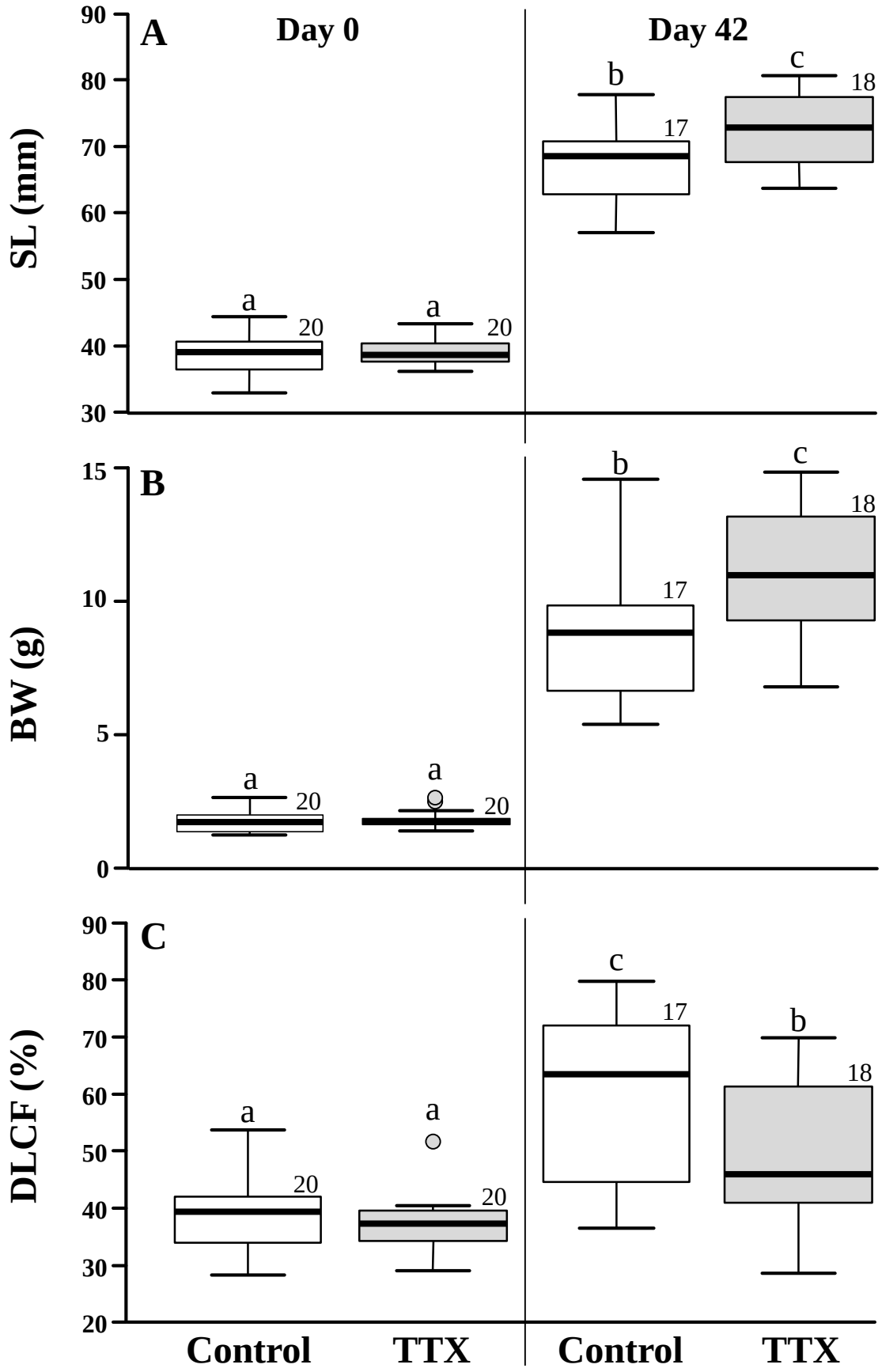

Fig. 2. Box plots of (A) SL (mm), (B) BW (g) and (C) DLCF (%) of the control and the TTX-treated group on Day 0 (initial day) and 42 (final day). Lower and upper box boundaries indicate 25th and 75th percentiles, respectively, line inside box median, lower and upper error lines 10th and 90th percentiles, respectively, and filled circles are the data falling outside 10th and 90th percentiles. Numbers indicate the number of fish employed. Different alphabetical letters above the boxes mean significant differences (a<b<c, pairwise comparison by least-squares means with Bonferroni correction after the Aligned Rank Transform for nonparametric factorial ANOVAs, $p<0.05$).

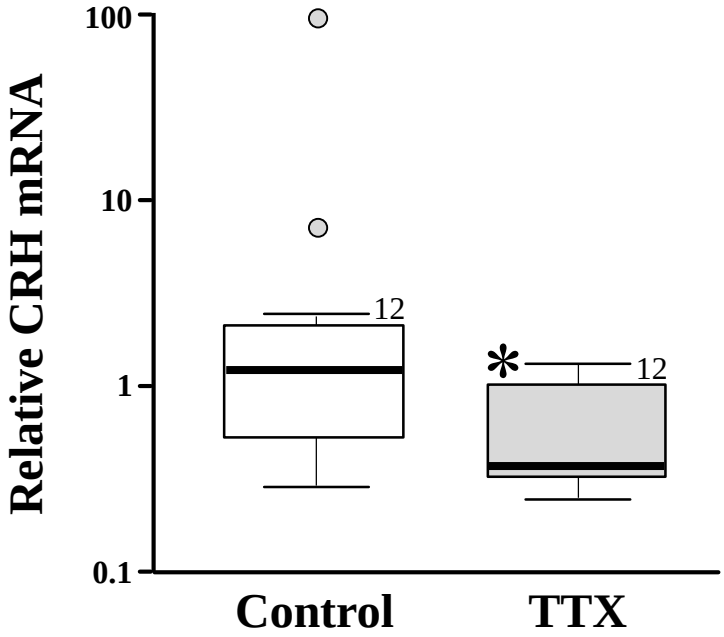

Fig. 3. Box plots of relative CRH mRNA expression in the brain of the control and the TTX-treated group on Day 42. Lower and upper box boundaries indicate 25th and 75th percentiles, respectively, line inside the box median, lower and upper error lines 10th and 90th percentiles respectively, and filled circles are the data falling outside 10th and 90th percentiles. Numbers indicate the number of fish employed. * (p<0.05) indicates the level of statistical difference between the two groups.

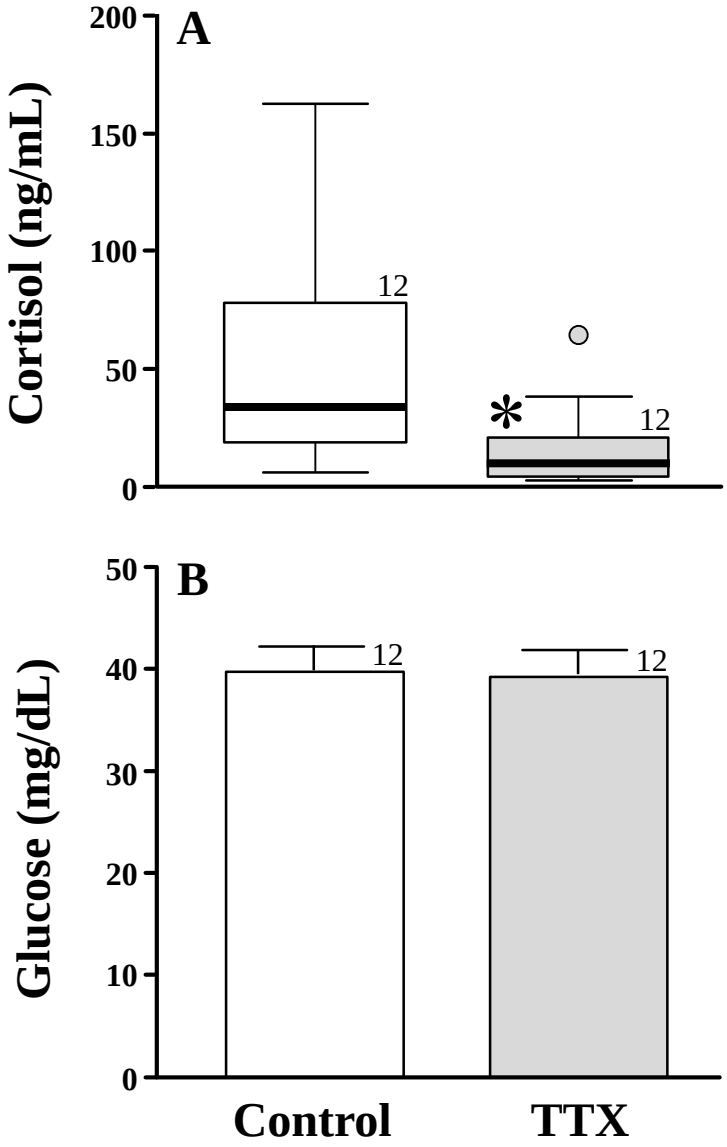

Fig. 4. (A) Box plots of plasma cortisol levels (ng/mL) and (B) bar plots of plasma glucose levels (mg/dL) of the control and the TTX-treated group on Day 42. (A) Lower and upper box boundaries indicate 25th and 75th percentiles, respectively, line inside the box median, lower and upper error lines 10th and 90th percentiles respectively, and filled circles are the data falling outside 10th and 90th percentiles. * (p<0.05) indicates the level of statistical difference between the two groups. (B) Each value is expressed as the mean and standard error (bar). Numbers indicate the number of fish employed.