Hormonal gene expression changes in the zebrafish caudal neurosecretory system under acute environmental challenges

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Abstract

The caudal neurosecretory system (CNSS) is neuroendocrine complex, found only in fishes, and suggested to be involved in osmoregulation and thermoregulation. Despite its physiological significance, the CNSS has been understudied, and its functional role remains enigmatic. While the zebrafish (*Danio rerio*) is a well-established model organism for functional studies, so far, the role of the CNSS has not really been investigated in this species. In the present study, we attempted to identify environmental factors whose variations induce changes in CNSS activity. Juvenile zebrafish were submitted to acute (2 h, 8 h, and 24 h) pH, salinity, and temperature challenges and the activity of the CNSS was quantified by measuring CNSS hormonal gene expression levels by quantitative PCR. Our findings revealed that temperature challenge affected the larger number of gene expression followed by salinity and pH challenge. This suggested that, in the zebrafish, the CNSS is involved in osmoregulation and thermoregulation, which is consistent with what has been reported in other species, and showed that the zebrafish is a relevant model to study the function of the CNSS.

1 Introduction

Facing numerous potential environmental changes inducing stress, fish are compelled to implement adaptive strategies to preserve homeostasis. In animals, maintaining physiological balance relies on mechanisms that regulate internal conditions to ensure a stable environment, despite external fluctuations. In vertebrate, one of the key players in maintaining homeostasis is the hypothalamo-hypophyseal neuroendocrine system, which play a central role for adapting to environmental variations and orchestrating appropriate physiological responses.

Although relatively little is known about it, fishes possess a second neuroendocrine complex located in the caudal part of the spinal cord, known as the caudal neurosecretory system (CNSS) (Rousseau et al., 2024). First described in the Japanese eel (*Anguilla japonica*) (Enami, 1955), the CNSS exist in all groups of jawed vertebrates (Gnathostomata) studied to date, except sarcopterygians (Enami and Ikai, 1955). In teleosts, the CNSS consists of a small population of large secretory neurons, known as Dahlgren cells (Dahlgren, 1914; Speidel, 1922), that project their non-myelinated axonal tracts toward a highly vascularized ventral expansion of the spinal cord called urophysis (Arnold-Reed et al., 1991; Bern, 1985; Fridberg and Bern, 1968). In this neurohaemal organ, secretory products are stored within axonal swellings of Dahlgren cells before being released into the bloodstream. Urophysis capillaries merge to form the caudal vein, which ensure its drainage and joins the portal-renal system. Beyond the kidney, the secretion products of the CNSS then access the systemic circulation, potentially interacting with renal cells, interrenal tissue, and the Corpuscles of Stannius (Bern et al., 1985).

The CNSS produces several peptide hormones, synthetized by Dahlgren cells and released by the urophysis. The two main secretion products of the CNSS were characterized from urophysial extract thanks to their pharmacological activity. Urotensin 1 (Uts1; also known as UI, and Urocortin 1 in mammals) is related to the Corticotropin-releasing hormone (Cardoso et al., 2016; Lovejoy and de Lannoy, 2013) and exhibits blood pressure-lowering properties (Lederis et al., 1982). Urotensin 2 (Uts2; also known as UII) is a cyclic neuropeptide with similarity with Somatostatin (Tostivint et al., 2013), that induces blood pressure elevation and smooth muscle contraction (Pearson et al., 1980). Additional peptide hormones were shown to be produced by the CNSS in different species. These include Corticotropin-releasing hormone (Crh) (Lu et al., 2004), Parathyroid hormone like hormone (Pthlh, also known as Parathyroid hormone-related protein) (Ingleton et al., 2002; Lu et al., 2017), Met-enkephalin (Yamada et al., 1988), and Staniocalcin (Greenwood et al., 2009). Further, the Arginine-vasopressin (Avp, also known as Vasotocin) and the Oxytocin (Oxt, also known as Isotocin) where found in the urophysis in different teleostean but whether these hormones are actually produced by the Dahlgren cells remain uncertain (Gozdowska et al., 2013; Lacanilao, 1972).

Since its first description, the CNSS has been proposed to play a role in fish adaptation to environmental changes, in particular in osmoregulation (Enami et al., 1956). Consistent with this idea, Uts1 and Uts2 were reported to modify ionic fluxes through skin (Marshall and Bern, 1981, 1979), intestine (Loretz et al., 1983; Mainoya and Bern, 1982) and urinary bladder (Loretz et al., 1983). Moreover, CNSS production is affected upon salinity challenge in different species. In the rainbow trout (*Oncorhynchus mykiss*), transfers from fresh water (FW) to sea water (SW) resulted in an increase of Uts1 immunoreactivity in Dahlgren cells but a reduction in urophysis, suggesting higher secretion in the bloodstream (Larson and Madani, 1996).

Similarly, increased levels of *uts1* and *crh* mRNA in daudal spinal cord were also reported following transfer to SW (Craig et al., 2005). As for Uts2, following transfer to SW, its secretion seems to be transiently reduced (2 h after transfer), but appears increased upon longer exposition (Larson and Madani, 1996). In the European flounder (*Platichthys flesus*), transfert from SW to FW increased *uts2* mRNA levels in the CNSS, while lowering plasma Uts2 concentration (Bond et al., 2002; Lu et al., 2006). In the same condition, *uts1* expression was transiently increased (8 h after transfer), but the urophysial content of Uts1 was reduced (Lu et al., 2019).

More recently, the CNSS was also suggested to be involved in thermoregulation. In the olive flounder (*Paralichthys olivaceus*), hypothermia led to increased *crh* and *uts2* mRNA levels in the CNSS, while hyperthermia increased Uts1-positive Dahlgren cells numbers and *uts1* mRNA levels [Yuan et al, 2019]. Further, in this species, the CNSS was found to directly respond to hypothermal challenge through TRP channels (Yuan et al. 2020), and this response seems to differ according to the temperature and the duration of the challenge (Yuan et al., 2021). Yet, in the rainbow trout, by contrast, cold stress did not induce any change in CNSS mRNA levels of *crh* and *uts1* (Craig et al., 2005).

Despite all these studies, the role of the CNSS remains largely enigmatic. The zebrafish is a well-known teleost model with unique advantages for functional studies. However, to date, only two study focused on the function of the CNSS in this species. One study reported that exposure of zebrafish embryos and young larvae (from 2 to 6 days post-fertilization, dpf) to seawater increased *uts2a* and *uts2b* expression at 6 dpf (Luo et al., 2014). Another study found that exposure to cold or hot stress downregulated the expression levels of *crh, uts1, uts2a* and *uts2b* (Luo et al., 2014). Nevertheless, as these experiments were conducted at very early

development stages, the significance of these variations remains difficult to evaluate. In the present study, in order to identify the physiological responses in which the CNSS plays a role in the zebrafish, we have attempted to determine environmental variations that induce changes in CNSS activity. We have investigated the effect of acute changes in water pH, salinity and temperature on CNSS hormonal gene expression levels. Our findings suggested that, in the zebrafish, the CNSS is involved in osmoregulation and thermoregulation, and showed that the zebrafish is a relevant model to study the function of the CNSS.

2 Materials and methods

2.1 Animals

Zebrafish (*Danio rerio*) used in this work were wildtype (Tübingen strain, from EZRC https://www.ezrc.kit.edu). All animals were bred in our facility and maintained according to standard procedure (Westerfield, 2007). Zebrafish embryos were obtained by natural spawning, collected from breeding tanks and kept in E3 embryo medium until 5 dpf. Larvae between 5 and 15 dpf were raised in water containing 5 g/l of sea salt (Instant Ocean) and fed *ad libitum* with rotifers. Both embryos and larvae were maintained in an incubator at 28.5°C under a 14-hour light/10-hour dark cycle. From 15 dpf onward, fish were kept in an Aquaneering standalone system with water at 26-27°C, pH 7-8, and conductivity 200-300 µS. Animals were maintained under a 14-hour light/10-hour dark cycle and fed once a day with live artemia and twice a day with dry food (Gemma Micro ZF, Skretting).

2.2 Ethic approval

This study was conducted in accordance with the European Communities Council Directive (2010/63/EU) and all procedures were approved by the Cuvier Institutional Ethics Committee at the Muséum national d'Histoire naturelle (APAFIS#35948). The study aimed to follow ARRIVE guidelines (Sert et al., 2020).

2.3 Experimental design for environmental challenge

The procedure was design to assess the impact of acute environmental variations. Three water parameters were tested: temperature, pH and salinity. For each parameter, three conditions (low, control and, high) and three time points (2 h, 8 h, and 24 h) were used.

Assays were conducted as follow: on the evening prior to the experiment, 63 sibling juvenile zebrafish (10 weeks) were randomly distributed in 9 groups, and placed in 1000 ml beakers (7 fish per beaker) containing 500 ml of control water (see next section). Fish were kept overnight in an incubator at 26°C with the same light-dark cycle as the facility. On the following morning, withing one hour after lights turned on, the control water was replaced with high-condition water (see next section) for three beakers, low-condition water (see next section) for three other beakers, and control water for the last three. Then, fish from one beaker for each water condition (high, low and control) were collected 2 h, 8 h and 24 h after the initial water change. At each time point, fish were rapidly euthanized using anesthetic overdose (tricaine methanesulfonate - MS222, 0.03% in high, low or control water) and sample were collected (see below). To maintain optimal water quality for the 24 h time point, the water was replaced with new high, low or control water before the second night.

2.4 Preparation of conditioned waters

In each experiment, the control water was prepared with deionized water supplemented with sea salt (Instant Ocean) at 0.15 g/l (conductivity ~250µS). The pH was maintained to 7.5 with HEPES (1 M) and the temperature was kept at 26°C.

For pH challenge, the high-condition water was at pH 9.0 and the low-condition water was et pH 6.0. Conditioned waters were obtained from control water with the pH adjusted with HCl or NaOH. For the salinity challenge, the high-condition water was 5 g/l and the and the low-condition water was 0.005 g/l (~15 µS). Conditioned waters were obtained from control water by adding sea salt or by dilution with deionized water. Finally, for temperature challenge, the high-condition water corresponded to 34°C and the low-condition water to 18°C. Here, control water was simply place into different incubator set to the corresponding temperature.

2.5 Sample collection

Following euthanasia, fish were dissected in ice-cold PBS. The anterior part was removed by cutting at the posterior end of the anal fin and the caudal fin was removed by cutting it at its base. Sample were placed individually in vials and snap-frozen in liquid nitrogen. All samples were stored at -80° C before further processing.

2.6 RNA extraction and real time quantitative RT-PCR

Total RNAs from samples were extracted using TRIzol Reagent (Invitrogen). Samples were directly immersed in 500 µL of TRIzol reagent, and homogenized using a TissueLyser II system (Qiagen). Following RNA extraction, sample were treated with DNAse I (Roche) to remove

potential contaminating genomic DNA, and then purified with phenol/chloroform extraction. The integrity of RNA was verified by electrophoresis on 1% agarose gel, and RNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher). Then, cDNAs were obtained using 500 ng of RNA with GoScript reverse transcriptase (Promega) and random primers (Promega). cDNA were diluted 10-fold prior to real-time PCR. Quantification was carried out by real-time PCR with specific primer pairs and PowerUp SYBR Green (Applied Biosystems) on a QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems). Primer pairs were validated with six serial dilutions of the corresponding cDNA, ensuring that CT variation was linear across dilutions and that melting curves showed a single peak. All primer sequences are indicated in Table 1. For all RT-qPCR experiments, reference genes were *lsm12b*, *mob4* (Hu et al., 2016) and *tdr7* (Vanhauwaert et al., 2014). Fold changes were calculated using the 2e-ΔΔCT formula.

2.7 Statistics and figure preparation

Prior to statistical analysis, outliers in gene expression data from RT-qPCR were identified and removed using interquartile range (IQR) method. Specifically, for each gene, and within each time and condition, samples with value more than 1.5 IQR below first quartile (Q1) or more than 1.5 IQR above third quartile (Q3) were considered outliers and excluded from data. Then, for each gene, and within each time and condition, statistical analysis was performed using two-sample Wilcoxon tests to compare values from high and low condition to the control.

Analyses were performed using the R software (R Core Team, 2021) along with the Tidyverse package (Wickham et al., 2019). Graphs were created using the ggplot2 package

(Wickham, 2016). Figures were prepared using Scribus software (www.scribus.net/) and Microsoft PowerPoint (Microsoft Corporation).

3 Results

In fish, the caudal neurosecretory system (CNSS) is thought to be involved in adaptation to environmental changes (Rousseau et al., 2024). In this study, we investigated whether acute changes in water pH, salinity or temperature resulted in modification of CNSS gene expression levels in zebrafish. For this, juvenile fish were exposed to three experimental conditions (control, high-condition, and low-condition) for each parameter and samples were collected after 2 h, 8 h and 24 h (Fig.1). In all cases, samples were used for RNA extraction and gene expression levels were measured using quantitative PCR. Genes assayed in this work were genes encoding neuropeptides previously reported to be produced by the CNSS (listed in Table 1). Note that we could not obtain specific primers for *stc1* nor *stc1l* and thus these two genes were not included in this work. Also, in all assays, the expression levels for *avp* and *crha* were too low to be analyzed and the corresponding data are not presented.

3.1 Effects of pH challenge.

Fish were exposed to water at pH 6 (low condition), pH 7.5 (control condition), and pH 9 (high condition). Figure 2 depicts the impact of these conditions on gene expression levels. In the 2 h challenge, *enka* was the only gene with modified expression levels, showing a clear reduction in the high condition compared to the control condition. At 8 h, the same tendency was observed for *enka* and two other genes had modified expression levels: *crhb* and *stc2b* with a reduction in the low condition. At 24 h, three genes had modified expression levels. *oxt*

was increased in low condition, *stc2b was* reduced the high condition and *uts2b* was reduced in both low and high conditions. Thus, overall, the pH challenge resulted in limited changes in gene expression.

3.2 Effects of salinity challenge.

Fish were exposed to water with salts at 5 ppm (low condition, see also methods), 150 ppm (control condition), and 5000 ppm (high condition). Gene expression levels under these conditions are illustrated in Figure 3. After 2 h, two genes, *oxt* and *uts1*, showed significant changes in expression levels compared to the control. o*xt* expression was increased in the low condition, with a similar tendency in high condition (p = 0.06), and *uts1* exhibited higher expression level in the high condition. With the 8 h challenge, we found that the expression level of *enka* was increased in the low condition. Consistent with our observation in the 2 h assay, our result suggested an increase of o*xt* expression level in the high condition (p = 0.08). Additionally, we also observed an increase in *uts2b* expression levels under the high condition (p = 0.05). Finally, in the 24 h challenge, we observed an upregulation of *stc2b* expression levels in the low salinity condition. We also noted a marked increase (approximately 2-fold) in *uts2b* expression levels in the low condition compared to the control. Interestingly, with the high condition, our results suggested increased expression levels for *uts2a* (p = 0.02), uts2b (p = 0.05), and *uts1* (p = 0.07). In conclusion, challenging zebrafish with salinity variation had limited effect on gene expression. Yet, it appeared to affect the expression levels of *uts1, uts2a uts2b* suggesting that, in zebrafish, Uts1 and Uts2 could be involved in the response to salinity variation as previously suggested by studies in various teleost species (Rousseau et al., 2024).

3.3 Effects of temperature challenge.

Fish were exposed to water at 18° C (low condition), 26° C (control condition), and 34° C (high condition). The effects on gene expressions levels are presented Figure 4. In the 2 h challenge, the expression levels of *enka* and *stc2a* were reduced in the high condition. This was also the case for *pthlha* in the low condition and, to a lesser extent, in the high condition ($p = 0.05$). Conversely, our data suggested increased expression levels of *stc2b* in the low condition ($p = 0.05$). Also, both *uts1* and *uts2a* expression levels appeared clearly increased in the high condition compared to the control. At 8h, most genes expression levels were altered in either the low or the high condition. In the low condition, *pthlha*, *pthlhb*, and *stc2a* were down-regulated compared to the control. This seemed to also be the case for *oxt* ($p = 0.05$). In the high condition, we observed increased expression levels for *crhb* and *uts2a*, and reduced expression levels for *oxt, enka*, *enkb* ($p = 0.07$). For the 24 h challenge, *enka*, *enkb, stc2a* and *stc2b* appeared downregulated in the high condition. Whereas, in the low condition, we observed an upregulation for *oxt*, *pthlha*, *stc2a* and *crhb* ($p = 0.05$). Finally, the expression of *uts1* was increased in the low temperature condition. Thus, overall, it appeared that in zebrafish, temperature is the environmental parameter that most significantly impact gene expression levels. Notably, both well-defined CNSS genes such as *uts1, uts2a* and *uts2b* and other genes were affected.

4 Discussion

Although first described 70 years ago (Enami and Ikai, 1955), the CNSS remains quite enigmatic. As a well-established genetic model organism for several decades, the zebrafish could help shed new light on the function of the CNSS. In this study, as a first step in this initiative, we attempted to identify environmental factors whose variations induce changes in CNSS activity. To ensure that the observed effects were physiologically relevant, the variations in water pH, salinity and temperature used in this study, were chosen in the range of conditions that zebrafish can encounter in their natural habitat (Arunachalam et al., 2013; Engeszer et al., 2007). One exception was the high salinity condition, where we used water with 5 g/l of sea salt, a concentration commonly employed as a treatment for skin infections in freshwater fish. Our results showed that CNSS activity, assayed by hormonal gene expression levels, is modified upon acute change in water temperature, salinity, and, to a lesser extent, pH. This suggest that, in the zebrafish, the CNSS is involved in osmoregulation and thermoregulation, which is consistent with what has been reported in other species, and shows that the zebrafish is a relevant model to study the function of the CNSS.

The implication of CNSS in osmoregulation has been proposed since its initial description by Enami, who found that osmotic stimuli induced histological changes of the CNSS in the pond loach (*Misgurnus anguillicaudatus*) (Enami, 1956). Since then, several studies have further supported this hypothesis in different species (Rousseau et al., 2024). Yet, in zebrafish, this had not been clearly shown since the only two reported studies (Jin et al., 2017; Luo et al., 2014) with this model used embryos and young larvae (between 2 and 6 dpf) in which the CNSS is likely not yet fully functional. Moreover, in these studies, mRNA were obtained from entire animal, raising questions about the source of the transcripts. Thus, our work provides

new evidence for a role of the CNSS in osmoregulation in zebrafish. Our result also suggested that the CNSS activity is influence by the pH. In our pH challenge, the overall salinity was identical in all three conditions and only the ionic composition was modified. Although the observed changes in CNSS gene expression were modest, our result suggest that the CNSS is also involved in the physiological adaptation to subtle change in water composition. This is in line with a study on brook trouts (*Salvelinus fontinalis)*, that reported that acidified water appears to stimulate release of Uts2 from the urophysis (Chevalier et al., 1986).

Recent studied have suggested that the CNSS is also involved in thermoregulation in the olive flounder (*Paralichthys olivaceus*) (Yuan et al., 2021, 2020, 2019). Earlier works in zebrafish had reported that temperature challenges modified expression levels of CNSS genes, but again this was done in embryo and young larvae (Jin et al., 2017; Luo et al., 2014). In our study, the temperature challenges resulted in the higher number of genes with modified expression levels. This suggest that, in the zebrafish, the CNSS could be more important for thermoregulation than for osmoregulation. Yet, while *uts1, uts2a* and *uts2b* are most likely specifically expressed in Dahlgren cells in the caudal part (Parmentier et al., 2008), this might not be the case for the other genes in our assay. Moreover, changes in temperature modify oxygen levels, which could also influence the activity of the CNSS. Thus, further analysis will be needed to confirm the implication of the CNSS in thermoregulation in zebrafish.

Because we meant to assay the effect of several parameters and conditions, we used juvenile zebrafish (10 weeks) which are easier to produce in numbers, compare to fully grown adults. However, the CNSS appears to developed relatively late during fish development (Fridberg and Bern, 1968; Oka et al., 1993 ; Cioni et al., 2000) and, as a consequence, we cannot rule out that in juvenile zebrafish, the CNSS is not yet fully mature. This could result in

lower gene expression levels and responses to challenges. Also, because we used animals before sexual maturity, we did not attempt to differentiate males and females. Nevertheless, it is possible that fine differences in expression levels and responses are already present at this stage. This would have increased variability between sample and diminished the power of our assay. It is thus clear that our results will have to be confirmed in adults.

In conclusion, our results provide the first evidence that the CNSS in zebrafish is involved in thermoregulation and osmoregulation. The zebrafish, as a model organism, is unique in the potential it offers for functional studies, notably thanks to efficient methods for CRISPR/Cas9-mediated genome editing and for transgenesis. Mutant lines, could be used to study the consequences of hormonal gene inactivation in control or modified water conditions. Since, some peptides are also produced outside of the CNSS, identifying that Dahlgren cell-specific regulatory sequence would enable, tissue specific gene inactivation to precisely investigate the function of CNSS products. Additionally, genetic cell ablation of Dahlgren cells could also be performed to revisit the effect of CNSS ablations. to and transgenesis. Thus, our work contributes to position the zebrafish a model of choice to study the role of the CNSS in fish homeostasis and should pave the way for further studies aimed at understanding the role of this neuroendocrine system, which is unique to fishes.

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Declaration of Competing Interests

The authors declare that they have no competing interests.

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Table 1

Gene name	Symbol	ZFIN ID
arginine vasopressin	avp	ZDB-GENE-030407-2
corticotropin releasing hormone a	crha	ZDB-GENE-070912-371
corticotropin releasing hormone b	crhb	ZDB-GENE-041114-75
proenkephalin a	penka	ZDB-GENE-030729-31
proenkephalin b	penkb	ZDB-GENE-030729-3
parathyroid hormone-like hormone a	pthlha	ZDB-GENE-050119-9
parathyroid hormone-like hormone b	pthlhb	ZDB-GENE-110411-206
stanniocalcin 1	stc1	ZDB-GENE-060825-79
stanniocalcin 1, like	stc1l	ZDB-GENE-040426-1521
stanniocalcin 2a	stc2a	ZDB-GENE-041008-14
stanniocalcin 2b	stc2b	ZDB-GENE-120203-5
urotensin 1	uts1	ZDB-GENE-041014-348
urotensin 2, alpha	uts2a	ZDB-GENE-040630-11
urotensin 2, beta	uts2b	ZDB-GENE-040630-10

Table 1. List of gene analyzed in this study. The ZFIN ID indicates the gene ID in the Zebrafish Information Network (ZFIN) database.

Figure 1

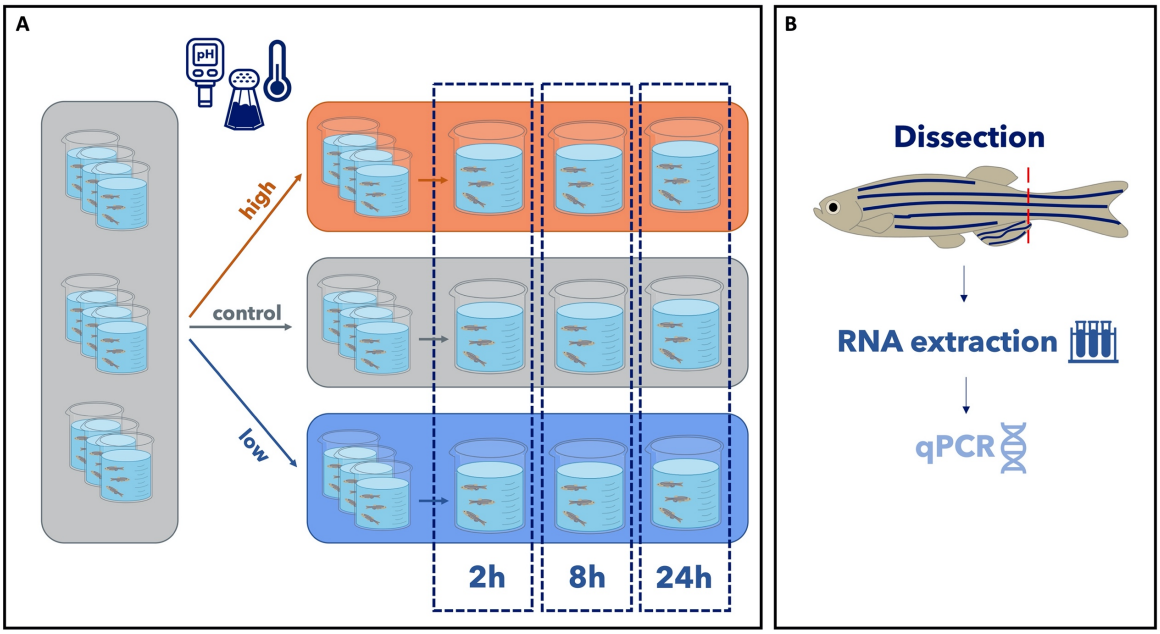

Fig 1. Overview of the experimental design. (A) The day prior to the experiment, juvenile zebrafish were randomly distributed into nine beakers with control water (7 fish per beaker). On the next morning, water was replaced with high, low or control condition water (see also method section). Sample were collected after 2 h, 8 h and 24 h. (B) Fish were dissected to only keep and the caudal part (minus the tailfin) and sample were processed for quantitative PCR analysis.

Figure 2

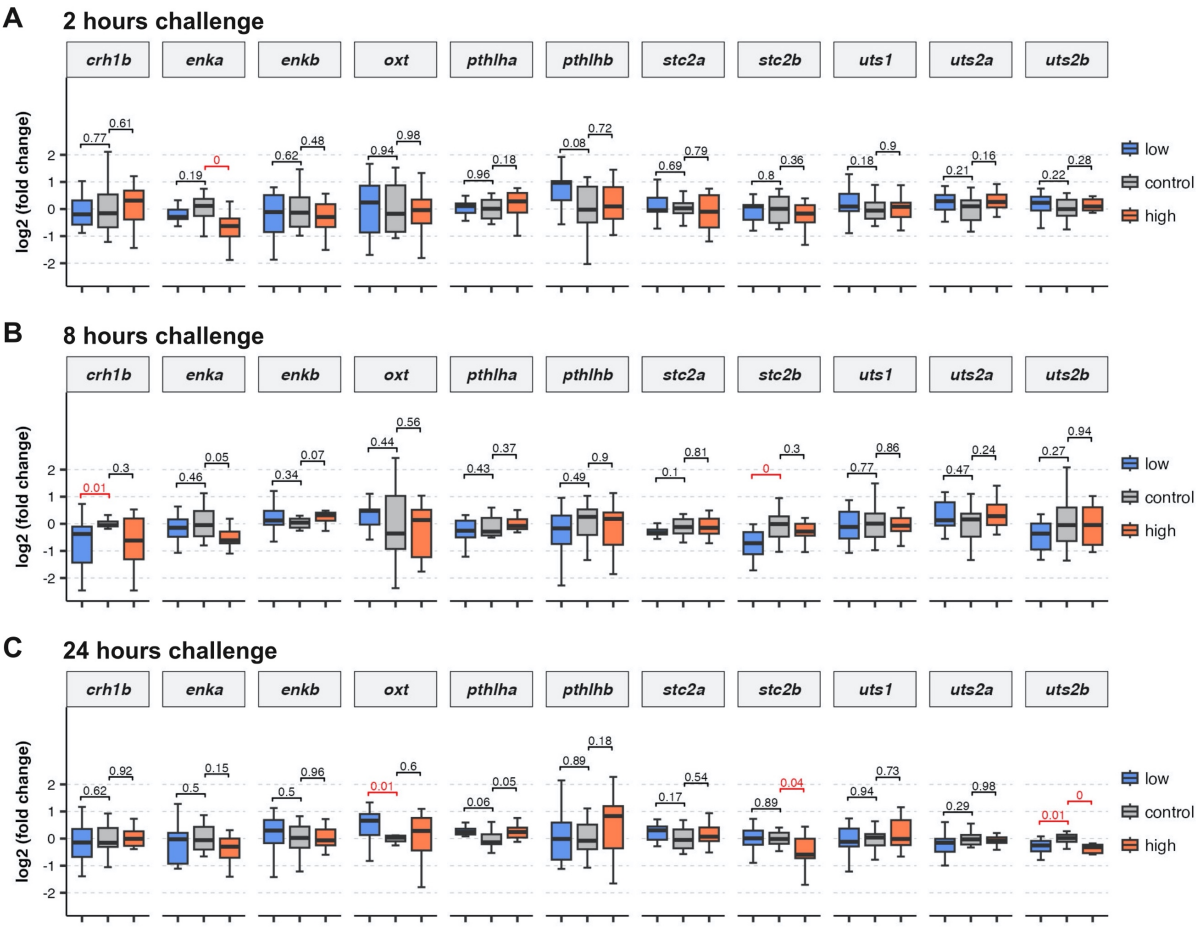

Fig 2. Gene expression changes following acute pH challenge. Expression levels of indicated genes after 2-hours (A), 8-hours(B) and 24-hours (C) pH challenge with low, control and high conditions. In all cases, results are expressed as log2 of fold change (ΔΔCT) compared to the control condition.

Figure 3

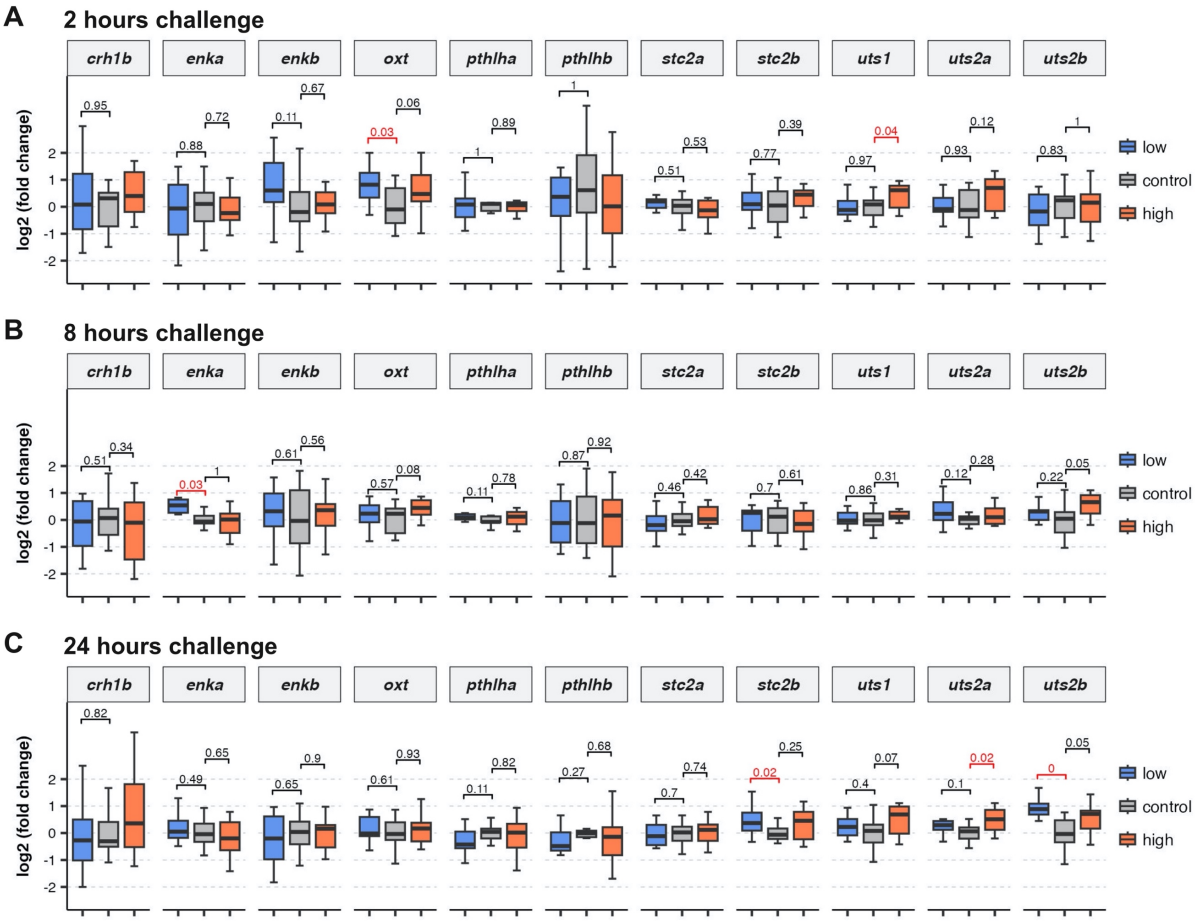

Fig 3. Gene expression changes following acute salinity challenge. Expression levels of indicated genes after 2-hours (A), 8-hours(B) and 24-hours (C) salinity challenge with low, control and high conditions. In all cases, results are expressed as log2 of fold change (ΔΔCT) compared to the control condition.

Figure 4

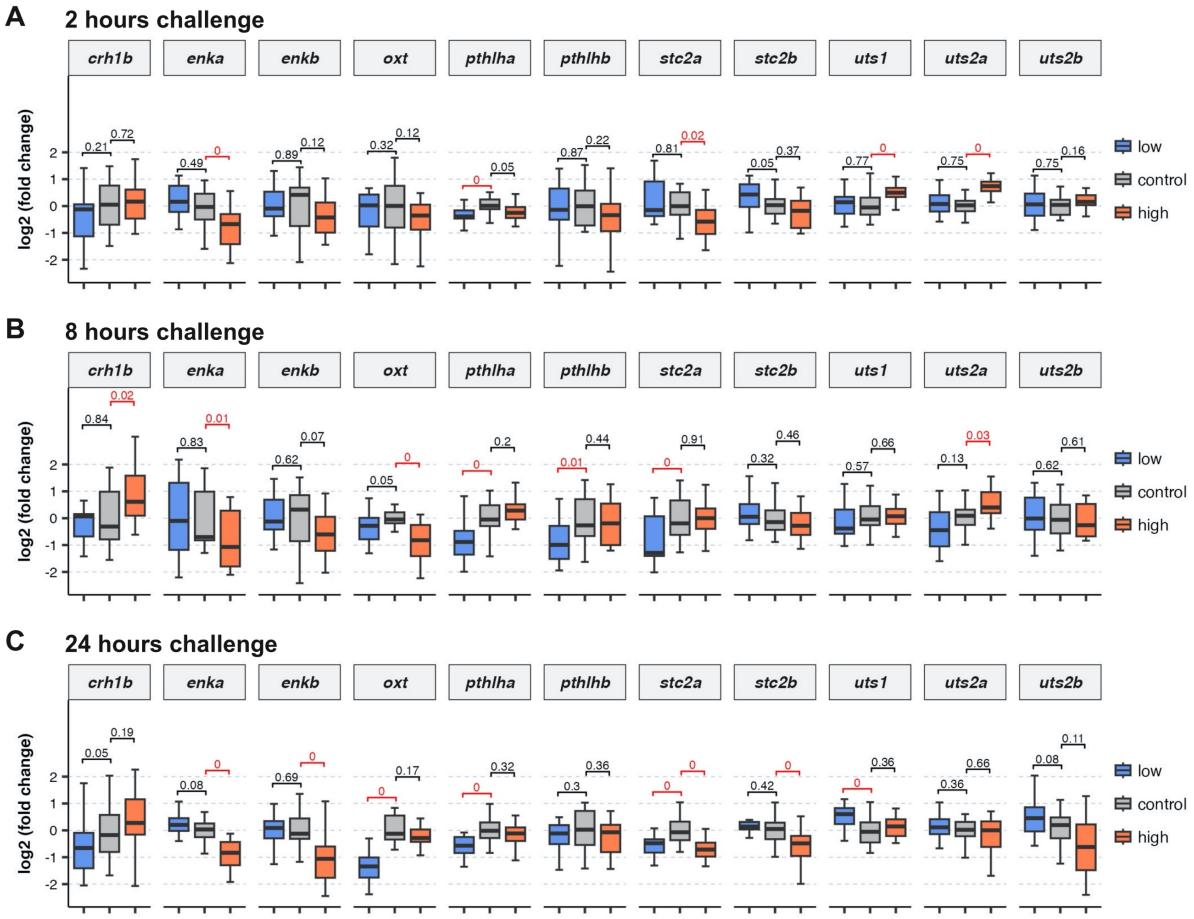

Fig 4. Gene expression changes following acute temperature challenge. Expression levels of indicated genes after a 2-hours (A), 8-hours(B) and 24-hours (C) temperature challenge with low, control and high conditions. In all cases, results are expressed as log2 of fold change (ΔΔCT) compared to the control condition.