Expression of endocrine genes in zebrafish larvae in response to environmental salinity

Kazuyuki Hoshijima and Shigehisa Hirose

Department of Biological Sciences, Tokyo Institute of Technology, 4259-B-19 Nagatsuta-cho, Midori-ku, Yokohama, Kanagawa 226-8501, Japan

(Requests for offprints should be addressed to S Hirose; Email: shirose@bio.titech.ac.jp)

Abstract

We tested the capability of the endocrine system in zebrafish to respond to environmental salinity challenges during larval stages. We reveal that the zebrafish larvae have a system in which several endocrine genes, including *atrial natriuretic peptide (anp)*, *renin*, *prolactin*, *growth hormone (gh)* and *parathyroid hormone 1 (pth1)*, respond at the transcription level to changes in environmental salinity and that the responses are gene specific. Both *anp* and *renin* are upregulated in larvae raised in dilute freshwater medium but are downregulated in concentrated medium. On the other hand, expression of

prolactin and *gh* is strongly enhanced in the dilute medium, but shows little or no change under higher salinity conditions. Interestingly, *PTH1* expression depends on Ca^{2+} concentration, as observed in mammals. Thus, taken together with the advantages of a model organism, including accessibility to genetic approaches, we propose that zebrafish larvae are useful for a comprehensive study of the regulatory mechanisms of the endocrine system in ionic and osmotic homeostasis.

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Introduction

Maintenance of constant ionic and osmotic states in body fluid is essential for survival of vertebrates, although the homeostatic strategies differ according to the external environment. Terrestrial tetrapods, which are constantly threatened with desiccation, principally retain water and salt. On the other hand, in freshwater, where ion concentrations are extremely low, teleost fish have to actively take up ions from the environment, to compensate for loss of ions down the concentration gradient, and must excrete excess water taken up by osmosis (Greenwell *et al.* 2003, Kirschner 2004, Evans *et al.* 2005). In contrast, in seawater, where ionic and osmotic strength are higher than in the body, fish must excrete excess ions and absorb water.

As in mammals, the endocrine system plays a central role in such homeostatic regulation in fish (McCormick & Bradshaw 2006). Many endocrine genes, including hormones and activating enzymes, have been identified in teleost fish and are known to function similar to mammalian genes with respect to ionic and osmotic homeostasis (McCormick 2001, Takei & Loretz 2005). However, it is also evident that some genes are functionally diverged to adapt to the aquatic environment. For example, atrial natriuretic peptide (ANP) primarily controls blood volume in mammals (Ruskoaho 1992, Loretz & Pollina 2000). ANP is secreted in response to an increase in blood volume and promotes excretion of water and Na^+ by the

kidney, and functions to inhibit Na^+ appetite and thirst, thus preventing further accumulation of Na^+ and water. On the other hand, ANP likely responds to the plasma Na^+ level, rather than blood volume, in eel, a euryhaline fish, which means it can adapt to both freshwater and seawater (Takei & Hirose 2002, Takei *et al.* 2006). During seawater adaptation, which tends to decrease blood volume but increase plasma Na^+ concentration, ANP is temporally secreted and prevents excess Na^+ absorption (Tsukada *et al.* 2005, Tsukada & Takei 2006). In zebrafish, the *anp* gene has been isolated and the expression is confined to the atrium during embryogenesis; the regulatory function remains unclear (Berdougo *et al.* 2003).

The renin–angiotensin system (RAS) also plays a critical role in blood volume control in mammals, although the regulatory direction is opposite that of ANP (Robertson 1993, Zhu & Herbert 1996). In response to a decrease in blood pressure, the system basically promotes dipsogenesis and reduces excretion of water and Na^+ by the kidney directly and indirectly. Renin, an enzyme that converts angiotensinogen to angiotensin I, is secreted from the juxtaglomerular cells of the kidney in response to a decrease in blood pressure (Gomez *et al.* 1988, Jones *et al.* 1990). In zebrafish, expression of *renin* has been shown in the juxtaglomerular cells, although no physiological function has been analyzed yet (Liang *et al.* 2004).

Prolactin and Growth Hormone (GH) are protein hormones that share a common structure and are mainly

expressed in the pituitary gland (Sakamoto & McCormick 2006). In mammals, function of Prolactin is best known for promotion lactation in the mammary gland (Riddle *et al.* 1933, Bole-Feysot *et al.* 1998, Gerlo *et al.* 2006). GH controls the growth of organisms through expression of insulin-like growth factor I (Kaplan 1999, Kopchick 2001). In teleost fish, both Prolactin and GH are involved in osmoregulation (Sakamoto & McCormick 2006). In a variety of freshwater and euryhaline teleosts, prolactin is required for adaptation to freshwater by promoting ion uptake and reducing water and ion permeability of the osmoregulatory surfaces, i.e. the gill, intestine, and kidney (Manzon 2002). On the other hand, GH has been viewed as seawater adapting in euryhaline species, as exogenous treatment of GH increased salinity tolerance (Smith 1956, Sakamoto *et al.* 1993). In zebrafish, both the *prolactin* and *gh* genes have been isolated and shown to be expressed in the pituitary gland during embryogenesis (Herzog *et al.* 2003, Liu *et al.* 2006).

Parathyroid hormone (PTH) is a regulator of Ca^{2+} homeostasis in mammals (Jüppner *et al.* 2000). A decrease in Ca^{2+} level in blood stimulates PTH secretion from the parathyroid glands. PTH accelerates bone resorption in osteoclasts and Ca^{2+} reabsorption in the kidney resulting in a recovery of Ca^{2+} levels in blood. It is not clear whether teleost fish have a similar PTH function, as they lack anatomically distinct parathyroid glands (Goodrich 1930, Hyman 1942, Copp 1969). However, PTH-related protein (PTHrP), derived from a common ancestor gene and sharing a receptor (PTH1R) with PTH, has been well studied in fish (Abbink & Flik 2006, Guerreiro *et al.* 2007), and the hypercalcemic function of PTHrP has been shown in sea bream (Abbink *et al.* 2004, Fuentes *et al.* 2006). Although their function remains unclear, recent studies have identified PTH homologs in zebrafish and in pufferfish (Gensure *et al.* 2004, Okabe & Graham 2004). The zebrafish *pth* genes, *pth1*, and *pth2* are predominantly expressed in the lateral line and in the ventral neural tube of the central nervous system (CNS; Hogan *et al.* 2005).

In this study, we examined the capability of the endocrine system in zebrafish larva to respond to an environmental

salinity challenge. Zebrafish have been extensively utilized as a model vertebrate for studies on embryogenesis and organogenesis, and the strengths of this model organism in studies an genetic approaches and the manipulation of gene expression and genome information, is that it is be adaptable for investigation of physiological functions, e.g. ionic and osmotic homeostasis (McGonnell & Fowkes 2006). Since zebrafish is a stenohaline freshwater fish that can adapt to salinity challenges in freshwater but not in seawater, we cultured the larvae in freshwater with different salinities and examined the endocrine response. Following investigation of the salinity range to which larvae can adapt, we investigated the transcriptional response of five endocrine genes: *anp*, *renin*, *prolactin*, *gh*, and *pth1*, to different salinities. We also analyzed the response of these genes to specific ions by altering the ionic composition of the medium.

Materials and Methods

Zebrafish culture

The wild-type zebrafish TL line was maintained as described earlier (Westerfield 1995). Fertilized eggs were incubated in 1× freshwater (1×FW:60 mg ocean salt (Rohtomarine, Rei-Sei, Tokyo, Japan) per 1 liter distilled water, pH 6.7–7.4) at 28.5 °C unless otherwise mentioned. Here, 1× FW was used as our standard freshwater medium and corresponds to ‘Fish Water’ as described in ‘The Zebrafish Book’ (Westerfield 1995). The concentration of major ions in and osmolarity of 1× FW are listed in Table 1. The animal protocols and procedures were approved by the Institutional Animal Care and Use Committee of Tokyo Institute of Technology.

Acclimation test of zebrafish larvae in freshwater with different salinities

In addition to 1× FW, we prepared 1/20× FW (3 mg ocean salt per liter, pH 7.2) as a dilute freshwater medium and 100× FW (6 g ocean salt per liter, pH 6.1–6.8) as a

Table 1 The concentration of the major ions and osmolarity in conditioned freshwater

	Ion/osmolyte (μM)						Osmolarity (mOsmol/l)
	Na ⁺	K ⁺	Ca ²⁺	Cl [−]	SO ₄ ^{2−}	Sorbitol	
1× FW	686	14.6	16.2	804	41.6	–	1.64
1/20× FW	34.3	0.730	0.810	40.2	2.08	–	0.0821
100× FW	68.6×10 ³	1.46×10 ³	1.62×10 ³	80.4×10 ³	4.16×10 ³	–	164
1/20× FW+supplement							
+ sorbitol	34.3	0.730	0.810	40.2	2.08	1.56×10 ³	1.64
+ NaCl	686	0.730	0.810	692	2.08	–	1.39
+ KCl	34.3	14.6	0.810	54.1	2.08	–	0.110
+ NaCl, KCl	686	14.6	0.810	706	2.08	–	1.41
+ CaCl ₂	34.3	0.730	16.2	71.0	2.08	–	0.128
+ NaCl, KCl, CaCl ₂	686	14.6	16.2	737	2.08	–	1.44

concentrated medium (Table 1). For long-term acclimation to $1/20\times$ FW and $100\times$ FW, 10–25 fertilized embryos reared in $1\times$ FW were dechorionated at 24 hours post-fertilization (hpf), rinsed with $1/20\times$ FW or $100\times$ FW, and cultured in 50 ml $1/20\times$ FW or $100\times$ FW in 100 mm Petri dish for 6 days respectively (Fig. 1A). For short-term exposure to $1/20\times$ FW and $100\times$ FW, 10–25 fertilized embryos reared in $1\times$ FW were dechorionated at 24 hpf, rinsed with $1\times$ FW and continuously cultured in 50 ml $1\times$ FW in 100 mm petri dish. At 6 days post-fertilization (dpf), the larvae were rinsed with $1/20\times$ FW or $100\times$ FW and cultured in 50 ml $1/20\times$ FW or $100\times$ FW in a 100 mm petri dish for 24 h respectively (Fig. 1A). To test the ability of recovery from exposure to dilute or concentrated freshwater, 24-hpf embryos were cultured in

$1/20\times$ FW or $100\times$ FW as described above, and were transferred back to $1\times$ FW on 6 dpf (=5 days post-acclimation) and cultured for 24 h. In a series of acclimation tests in $1/20\times$ FW with particular supplements (Table 1), the dechorionated embryos were cultured in the conditioned water for 6 days as described for long-term acclimation to $1/20\times$ FW. As a control, dechorionated embryos were continuously cultured in $1\times$ FW until 7 dpf. During the incubation, dead larvae were removed every day. On 7 dpf, living larvae were counted and the morphology was recorded and photographed with MZ16F stereomicroscope (Leica, Germany). We note that the zebrafish larvae were not fed during these tests, and we have verified that the larvae are able to survive without feeding at least until 10 dpf.

Figure 1 Larval development of zebrafish cultured in different salinity conditions. (A) Schematic representation of acclimation protocols. $1\times$ FW (shaded box): standard freshwater medium, $1/20\times$ FW (open box): 20-fold diluted freshwater medium, $100\times$ FW (solid box): 100-fold concentrated freshwater medium (see Materials and Methods, and Table 1). The length of culture in each medium is represented in days post-fertilization (dpf) across the top of the schematic. We note that prior to the acclimation test, zebrafish embryos developed for 24 h in the chorion in $1\times$ FW medium (hatched box). (B) Viability of zebrafish larvae cultured in $1/20\times$ FW or $100\times$ FW under long- (24 hpf to 7 dpf) or short-term (6 to 7 dpf) acclimation protocols. (C) Viability of zebrafish larvae post-long-term acclimation in $1/20\times$ FW or $100\times$ FW, and after readaptation. Open and shaded boxes indicate the percentage of living larvae developing normally and those lacking swimbladder respectively. Numbers in parentheses represent the number of larvae examined in each condition.

Quantitative PCR

At the end of the acclimation tests, for each condition, larvae ($n=10-25$) in a dish were collected and total RNA was extracted with the RNeasy Lipid Tissue Kit (Qiagen) followed by the RNeasy MiniElute Cleanup Kit (Qiagen) according to the manufacturer's instruction. The RNA was dissolved in 14 μ l diethyl pyrocarbonate-treated water, and its concentration was measured spectrophotometrically at 260 nm. Single-stranded cDNA was prepared from 500 ng total RNA with oligo (dT) primer in a 20 μ l reaction volume using Superscript III First-Strand System (Invitrogen) and finally diluted in 40 μ l diethyl pyrocarbonate-treated water. Real-time PCR was set up using 2.5 μ l cDNA, 12.5 μ l 2 $\times$ SYBR Green Master Mix (Applied Biosystems, ABI, Foster City, CA, USA), and 50 nM each of forward and reverse primers in a total volume of 25 μ l. The real-time PCR was run for 45 cycles on the 7500 Real-Time PCR System (ABI) according to the manufacturer's protocol. Primers for each target gene were designed to generate a product around 55 bp in size, and their efficiency was validated by graphical representation of slope and fluorescence intensity/cycle number (see Table 2 for primer sequence). The amount of cDNA in a sample was calculated by comparing the threshold cycle (C_t) in the sample against a standard curve generated from a series of diluted cDNAs that were prepared from RNA obtained from larvae raised in standard freshwater (1 $\times$ FW) with primers for each target. The amount of ribosomal protein L9 (*rpl9*) in each sample was used for normalization. The resultant value in each condition was obtained from at least three independent acclimation experiments and calibrated to that in 1 $\times$ FW and represented on a logarithmic scale. We also used the *gapdh* gene (GenBank accession number AY818346) as a normalization control for quantitative PCR and obtained results consistent with the *rpl9* control (data not shown).

Whole mount in situ hybridization

Whole mount *in situ* hybridization was performed as previously described with brief modifications (Novak & Ribera 2003, Esaki *et al.* 2007), using a digoxigenin-labeled RNA probe for *anp* corresponding to a 365-bp fragment of the cDNA (Genbank accession number NM_198800;

nucleotide (nt) 104–468), *renin* corresponding to a 591-bp fragment of the cDNA (NM_212860; nt 537–1127), *prolactin* corresponding to a 550-bp fragment of the cDNA (NM_181437; nt 214–763), *gh* corresponding to a 451-bp fragment of the cDNA (NM_001020492; nt 213–663), *pth1* corresponding to a 571-bp fragment of the cDNA (NM_212950; nt 54–624), or *podocin* corresponding to a 637-bp fragment of the cDNA (AY956356; nt 706–1342). A fluorescein-labeled RNA probe was also prepared for *renin* using a cDNA fragment identical to the digoxigenin-labeled probe. *In situ* hybridization with single digoxigenin-labeled probe was carried out using the wild-type TL strain. After staining with NBT/BCIP color development substrate (Promega), larvae were depigmented and permeabilized extensively by incubation with 20% H₂O₂ in PBT (137 mM NaCl, 27 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, 0.1% Tween-20, pH 7.4) overnight or longer. For double *in situ* hybridization, 7-dpf albino larvae were hybridized simultaneously with a digoxigenin-labeled *podocin* probe and a fluorescein-labeled *renin* probe. Following incubation with anti-digoxigenin Fab fragment conjugated with alkaline phosphatase (diluted 1:4000, Roche), *podocin* signal was first developed in red using SIGMAFAST Fast Red TR/Naphthol AS-MX (Sigma). Subsequently, larvae were incubated with anti-fluorescein Fab fragment conjugated with alkaline phosphatase (diluted 1:2000, Roche) and *renin* signal was developed in purple with NBT/BCIP color development substrate.

Results

Larval development of zebrafish in freshwater with different salinities

Prior to examining the endocrine response in zebrafish larvae to environmental salinity, we examined the range of salinities in which the larvae were able to survive. In this study, standard freshwater (1 $\times$ FW) was the medium used for the routine culture of zebrafish embryos/larvae. We also prepared 1/20 $\times$ FW and 100 $\times$ FW, and transferred the larvae to these media at different developmental points (Fig. 1A). When larvae were transferred from 1 $\times$ FW to 1/20 $\times$ FW at 24 hpf and

Table 2 Primers used for quantitative PCR

	GenBank accession no.	Forward primer	Reverse primer	Product size (bp)	Amplification efficiency (%)
Target gene					
<i>anp</i>	NM_198800	AGCAACATGGCCAAGCTCAAG	CAGGGCCTCCTCAAACCTGC	51	89.4
<i>renin</i>	NM_212860	TGAAGTGGCAGAAGGAGGGTATA	GCAACAGTGGGCAACAACCT	63	85.4
<i>prolactin</i>	NM_181437	GGCCTGGAGCACGTCGTA	ACGGGAGAGTGGACAGGTTGT	61	98.8
<i>gh</i>	NM_001020492	CACCTGAGAACTGGTGGACCTG	TCCCTTGATGAGCACGCTG	52	86.5
<i>pth1</i>	NM_212950	AGAAGCAAACGGGCAGTGA	TGAACACCGAGATTATGCATCAG	62	86.6
<i>rpl9</i>	NM_001003861	TCCGCATGAGGCAAGGAG	TCGTCTTTTGGAGCAGCAGACA	52	87.8

cultured for 6 days, viability was similar to that of control larvae ($>95\%$ viability), which were continuously cultured in $1\times$ FW for 7 days (Fig. 1B). However, we observed that most of the larvae cultured in $1/20\times$ FW did not develop the swimbladder, which usually appears by 5 dpf (Figs 1B and 2A and B). Otherwise, larval morphology appeared normal. These results suggest that $1/20\times$ FW is likely to be close to the lower limit of ionic concentration in which zebrafish larvae can survive, although development is affected. Conversely, when zebrafish larvae were transferred from $1\times$ FW to $100\times$ FW at 24 hpf and cultured for 6 days, the larvae survived as well as controls without any detectable defects in morphology (Figs 1B and 2C).

Next, we examined the effects of short-term acclimation to $1/20\times$ FW and $100\times$ FW (Fig. 1A). The zebrafish larvae that were transferred from $1\times$ FW to $1/20\times$ FW at 6 dpf and cultured for 24 h exhibited high viability with well-developed swimbladders (Fig. 1B). Interestingly, when the larvae were

transferred to $100\times$ FW at 6 dpf and cultured for 24 h, viability was reduced to approximately two-thirds, although the survivors did not show any obvious defects in morphology (Fig. 1B). These results suggest that in $100\times$ FW 6-dpf-larvae cannot quickly adapt the saline challenge, but require a longer acclimation time, beginning at a younger stage.

We also analyzed effects of readaptation to the standard freshwater condition from the dilute and concentrated media (Fig. 1A). Following culture in $1/20\times$ FW for 5 days (24 hpf to 6 dpf), zebrafish larvae were transferred back to $1\times$ FW and cultured for 24 h. Readaptation did not affect viability but did result in the development of the swimbladder in a significant number of larvae (Figs 1C and 2D). This recovery was solely dependent on readaptation, as most of the larvae cultured in $1/20\times$ FW continuously for 6 days (24 hpf to 7 dpf) did not develop the swimbladder (Fig. 1C). These results suggest that the morphological defect caused by culture in $1/20\times$ FW is reversible and quickly recovered under standard salinity

Figure 2 Morphology of zebrafish larvae cultured in different salinity conditions. (A–D) Morphology of 7 dpf zebrafish larvae cultured in $1\times$ FW (A), acclimated in $1/20\times$ FW (long term) (B), acclimated in $100\times$ FW (long term) (C), and acclimated in $1/20\times$ FW for 5 days and then in $1\times$ FW for 24 h (readaptation) (D). (E–J) Morphology of 7-dpf zebrafish larvae cultured in $1/20\times$ FW + sorbitol (E), in $1/20\times$ FW + NaCl (F), in $1/20\times$ FW + KCl (G), in $1/20\times$ FW + NaCl, KCl (H), in $1/20\times$ FW + CaCl_2 (I), and in $1/20\times$ FW + NaCl, KCl, CaCl_2 (J). Arrowheads indicate the swimbladder (missing in B, E, G, and H). Scale bars, 1 mm.

Table 3 PO₂/PCO₂ in conditioned freshwater. Mean and S.E.M. values were obtained by triplicate measurements

	In stock ^a	In cultured dish ^b
Medium		
1× FW	25.31±0.13	27.28±2.25
1/20× FW	25.35±0.44	24.49±0.20
100× FW	25.91±0.56	23.55±0.21

^aPO₂ (mmHg) and PCO₂ (mmHg) in stock solution of each medium (at 28.5 °C). were measured by Radiometer ABL505 (Radiometer, Copenhagen Denmark) and PO₂/PCO₂ was calculated.
^bPO₂ (mmHg) and PCO₂ (mmHg) in Petri dishes in which zebrafish larvae (n=20) were cultured in each medium for 6 days (24 hpf to 7 dpf) were measured by Radiometer ABL505 (Radiometer) and PO₂/PCO₂ was calculated.

conditions. This finding also supports the notion that 1/20× FW is close to the lower limit of salinity in which zebrafish larvae can survive. Readaptation from 100× FW to 1× FW did not show any obvious effects on viability or morphology. Inflation of the swimbladder possibly depends on ratio of PO₂ to PCO₂ (PO₂/PCO₂) in different media (Randall *et al.* 2002). However, PO₂/PCO₂ did not show salinity-dependency among our conditioned media (Table 3).

Endocrine response to environmental salinity

Using 1/20× FW and 100× FW as our dilute and concentrated media respectively, we analyzed the expression level of

endocrine genes in zebrafish larvae cultured under different salinity conditions. From a preliminary screen of more than 20 endocrine genes, including hormones, activating enzymes or receptors, we found that *anp*, *renin*, *prolactin*, *gh*, and *pth1* appeared to respond to the environmental salinity challenge (data not shown). We further analyzed the response of these five endocrine genes. We first confirmed the previously reported expression of these genes at 7 dpf (Fig. 3; Berdougo *et al.* 2003, Herzog *et al.* 2003, Liang *et al.* 2004, Hogan *et al.* 2005). When zebrafish larvae were cultured in 1/20× FW for 6 days (24 hpf to 7 dpf), the expression level of *anp* and *renin* increased several fold when compared with the expression in larvae cultured in standard freshwater (Fig. 4). Remarkably, the expression of *prolactin* and *gh* was elevated to nearly 100-fold or more in 1/20× FW. *pth1* expression also increased similarly to *anp* and *renin*. On the other hand, in larvae cultured in 100× FW for 6 days, the expression of *anp*, *renin*, *prolactin*, and *pth1* was significantly reduced, while *gh* remained constant. Thus, the endocrine genes in zebrafish larvae respond at the transcriptional level to environmental salinity challenges.

When zebrafish larvae were acclimated in 1/20× FW for 24 h (6–7 dpf), upregulation of *prolactin* and *gh* was observed, although the elevation of each did not reach the levels observed after long-term acclimation (Fig. 4). Changes in *anp*, *renin*, and *pth1* expression were not significant after short-term acclimation. In contrast, after acclimation in 100× FW for 24 h, we observed reduced expression of *anp*, *renin*, *prolactin*, and *pth1*, but not *gh*.

Figure 3 Expression of *anp*, *renin*, *prolactin*, *gh*, and *pth1* in 7 dpf larvae. (A–F) Tissue-specific distribution of *anp* (A, ventral view), *renin* (B and C, lateral view), *prolactin* (D, dorsal view), *gh* (E, dorsal view), and *pth1* (F, lateral view) was analyzed in zebrafish larvae at 7 dpf by whole mount *in situ* hybridization. Arrows indicate *anp* expression in the atrium (A), *renin* expression in the juxtaglomerular cells (B and C), *prolactin* and *gh* expression in the pituitary gland (C and D respectively), and *pth1* expression in a portion of the ventral neural tube of the CNS (E). In panel (C), *renin* was co-stained with a glomerulus marker, *podocin* (GenBank accession number, NM_001018145). *Podocin* (red, arrowhead) and *renin* (purple, arrow) were expressed adjacent to each other, indicating that *renin* expression was in the juxtaglomerular cells. We note that except in (C) larvae are wild-type TL strain that have been depigmented by 20% H₂O₂ after staining. In panel (C), we used an albino strain as the depigmentation process could cause the red staining to fade. Scale bars, 0.5 mm.

Figure 4 The expression level of endocrine genes depends on environmental salinities. Expression level of *anp*, *renin*, *prolactin*, *gh*, and *pth1* was measured in zebrafish larvae after long- and short-term acclimation to 1/20× FW or 100× FW. The expression level of each gene was normalized by comparison with the expression of RPL9. The mean value ± S.E.M. in each condition was obtained from replicate acclimation experiments ($n=3-14$) and were calibrated to expression in 1× FW and represented on a logarithmic scale. S.E.M. value of each gene in 1× FW was also normalized and plotted as an error bar. Asterisks indicate a significant difference from the expression level in 1× FW as evaluated by a Mann–Whitney *U*-test (** $P<0.01$, * $P<0.05$).

Finally, we also analyzed expression level of these genes after readaptation to 1× FW from 1/20× FW and 100× FW. Following readaptation to 1× FW for 24 h after 5 days culture in 1/20× FW, the expression of *anp*, *renin*, and *pth1* was reduced to levels seen in standard conditions (Fig. 5). The expression level of *prolactin* and *gh* after readaptation from 1/20× FW appeared higher than the standard level but was not statistically significant. We also observed that the expression level of these genes returned to standard levels when larvae were readapted to 1× FW for 24 h after 5 days culture in 100× FW (Fig. 5).

Effects on larval development of freshwater with aberrant ionic compositions

Next, we addressed whether the endocrine genes analyzed above exhibit any osmolarity- and ion-specific responses by preparing freshwater in which the osmolarity and ionic compositions were modified with an organic osmolyte or specific ion (Table 1). First, we examined the effects of reconstituted freshwater on viability and morphogenesis of zebrafish larvae. When we cultured larvae for 6 days (24 hpf to 7 dpf) in 1/20× FW with sorbitol, in which the osmolarity was equal to 1× FW but the ionic strength remained 1/20×, viability was not affected (Fig. 6). However, most larvae did not develop a swimbladder, indicating that

restoration of osmolarity is not sufficient for proper development of the swimbladder (Figs 2E and 6). Interestingly, in 1/20× FW with NaCl, in which Na^+ was added so as to be equal to 1× (Cl^- and osmolarity was also recovered to 0.85×), while other ions remained at 1/20× concentration, the larvae exhibited severe morphological defects accompanied by a reduction of viability. When cultured in 1/20× FW with NaCl for 6 days, not only was the viability reduced to 63% but also all of the survivors had a curled body and exhibited severe edema of the chest and abdomen (Figs 2F and 6). These results indicate that normal Na^+ concentration becomes toxic for zebrafish larvae if it is unbalanced with respect to other ions. On the other hand, supplementation of K^+ did not show such toxicity: among larvae cultured in 1/20× FW with KCl, the viability was high and ~50% of the larvae developed normally including the swimbladder (Figs 2G and 6). Furthermore, addition of KCl to 1/20× FW with NaCl neutralized the toxicity caused by elevated Na^+ . When larvae were cultured for 6 days in 1/20× FW with NaCl and KCl, in which both Na^+ and K^+ were raised to 1× concentration, viability, and morphology were recovered, although substantial numbers of the larvae still lacked the swimbladder (Figs 2H and 6). In contrast to supplementation with Na^+ and K^+ , supplementation of Ca^{2+} promoted normal development. In 1/20× FW with CaCl_2 , in which Ca^{2+} was equal to 1× but other ions remained 1/20× concentration

Figure 5 Expression level of endocrine genes after readaptation to standard freshwater medium, following culture in dilute or concentrated media. Expression level of *anp*, *renin*, *prolactin*, *gh*, and *pth1* was measured in zebrafish larvae cultured in 1/20× FW or 100× FW for 5 days and then in 1× FW for 24 h. The expression level of each gene obtained from replicate experiments ($n \geq 3$) was normalized, calibrated and represented as described in Fig. 4. We note that evaluation by Mann–Whitney *U*-test did not show any significant differences in expression levels for any gene between 1× FW and readaptation conditions.

(Cl^- was $\sim 1/10\times$), larvae developed normally with high viability (Figs 2I and 6). In 1/20× FW with NaCl, KCl, and CaCl_2 , the larvae also developed normally (Figs 2J and 6). These results suggest that it is likely the limited Ca^{2+} in 1/20× FW that causes the abnormal development of zebrafish larvae.

Figure 6 Viability of zebrafish larvae in dilute freshwater with supplementation of an organic osmolyte or specific ions. Viability of 7-dpf zebrafish larvae was analyzed after culturing for 6 days (24 hpf to 7 dpf) in 1/20× FW with a variety of supplements (see Materials and Methods, and Table 1). Open and shaded boxes indicate the percentage of living larvae developing normally and those lacking swimbladder respectively. Solid box indicates the percentage of larvae developing abnormally with severe edema. Numbers in parentheses represents the number of larvae examined in each condition.

Endocrine response to ambient salinity with aberrant ionic composition

We analyzed the expression level of *anp*, *renin*, *prolactin*, *gh*, and *pth1* in the modified freshwater media. In larvae cultured in 1/20× FW with sorbitol for 6 days (24 hpf to 7 dpf), expression level of these genes was very similar to expression in 1/20× FW (Fig. 7). This is consistent with our observation that supplementation of 1/20× FW with sorbitol did not alter viability or morphology. In 1/20× FW with NaCl, all genes except *renin* were further upregulated when compared with expression in larvae cultured in 1/20× FW. In 1/20× FW with KCl, expression of *renin*, *prolactin*, *gh*, and *pth1* was significantly downregulated from levels seen in 1/20× FW. In 1/20× FW with NaCl and KCl, the expression level of these genes was similar to expression in 1/20× FW. Only *pth1* expression was significantly reduced from levels seen in 1/20× FW. The upregulation of *prolactin*, *gh*, and *pth1* by supplementation with NaCl is somehow neutralized by the addition of K^+ . In 1/20× FW with CaCl_2 , consistent with the promotion of normal development, expression of all the endocrine genes except *anp* was significantly reduced when compared with expression in 1/20× FW. We observed that expression of these genes was further downregulated in 1/20× FW with NaCl, KCl and CaCl_2 . Thus, the endocrine genes in zebrafish larvae are able to respond to environmental salinity, and each gene has a different response to individual ions.

Discussion

In this report, we examine the response of the zebrafish endocrine system to environmental salinity challenges. In teleost fish including zebrafish, ionic and osmotic homeostasis of the body fluid is directly affected by environmental salinity because the plasma contacts the environment through respiratory epithelia. Therefore, as a homeostatic strategy, the endocrine system in teleost fish must respond to the environmental salinity. Furthermore, by manipulating the environmental salinity, we can perturb the ionic and osmotic balance of the body fluid in teleost fish and examine the endocrine response, as a model for studying homeostasis. Using freshwater with altered salinities, we have detected a transcriptional response of several endocrine genes in zebrafish larvae to the external salinity. Interestingly, *anp* and *renin* were regulated in the same direction: they were upregulated in the dilute condition (1/20× FW) and downregulated after culture in the concentrated medium (100× FW; Fig. 4). This response is quite different from that observed in mammals where ANP and Renin/RAS have opposite effects on blood volume control: ANP responds to hypervolemia and promotes excretion of water and Na^+ , while Renin responds to hypovolemia and RAS promotes reabsorption of water and Na^+ (Ruskoaho 1992, Robertson 1993). These differences between teleosts

Figure 7 Expression level of endocrine genes in zebrafish larvae cultured in 1/20× FW with a variety of supplements. Expression level of *anp*, *renin*, *prolactin*, *gh*, and *pth1* was measured in zebrafish larvae cultured for 6 days (24 hpf to 7 dpf) in 1/20× FW with a variety of supplements (see Materials and Methods, and Table 1). The expression level of each gene obtained from replicate experiments ($n \geq 3$) was normalized, calibrated and represented as described in Fig. 4. Asterisks and cross indicate a significant difference from the expression level in 1× FW and 1/20× FW respectively, as evaluated by a Mann-Whitney *U*-test, (** $P < 0.01$, * $P < 0.05$, † $P < 0.05$).

and mammals likely reflect the relationship between blood volume and plasma Na^+ control. In mammals, volume control is usually in the same direction as plasma Na^+ control, as mentioned above. In teleosts, however, the direction of volume control is opposite of plasma Na^+ control. For example, in freshwater, hypervolemia and hyponatremia is the result of the influx of excess water and loss of Na^+ according to the osmotic gradient. In further diluted salinity conditions, i.e. 1/20× FW, hypervolemia and hyponatremia would be more extreme. We speculate that *anp* expression in 1/20× FW may increase to respond to severe hypervolemia and promote excretion of excess water, similar to ANP function in mammals, while the increase in renin expression may be a response to the severe hyponatremia leading to Na^+ absorption as in mammals. The expression of *anp* and *renin* in 1/20× FW supplemented with NaCl supports this hypothesis. In 1/20× FW with NaCl, zebrafish larvae formed severe edema in the chest and abdomen, likely due to uncontrollable hypervolemia, and *anp*, but not *renin*, expression was further elevated when compared with expression in 1/20× FW (Fig. 7, 1/20× FW with NaCl), indicating that *anp* rather than *renin* responded to

the severe hypervolemia. On the other hand, reduction of renin expression, though not significant, may be responding to the increase in Na^+ from 1/20× to 1× concentration. Thus, in zebrafish, ANP, and Renin/RAS may be the volume-regulating and Na^+ -regulating hormones during freshwater adaptation, respectively. However, this notion cannot be generalized in teleosts as it is evident that ANP is likely to be the Na^+ -regulating and seawater-adapting hormone in euryhaline eels (Takei & Hirose 2002, Takei *et al.* 2006). The difference may be species specific, although further investigation in other teleosts, especially in stenohaline freshwater fish, will be required to clarify the action of ANP and Renin/RAS in freshwater adaptation.

Expression of *prolactin* was strongly induced in zebrafish cultured in 1/20× FW, which is consistent with the osmoregulatory function during freshwater adaptation observed in other teleost fish (Burden 1956, Pickford & Phillips 1959, Horseman 1987, Manzoni 2002). Surprisingly, like *prolactin*, *gh* expression was also elevated in 1/20× FW, which is in contrast to the function of GH as seawater-adapting hormone in some euryhaline teleosts (Smith 1956, Sakamoto *et al.* 1993). Possibly, GH functions differently in the stenohaline zebrafish than in the euryhaline teleosts.

Alternatively, more GH may be required for larval growth including bone formation when Ca^{2+} availability is limited in $1/20 \times \text{FW}$, leading to the higher expression level. Supporting this hypothesis, the expression level of *gh* decreased upon addition of Ca^{2+} to $1/20 \times \text{FW}$ (Fig. 7, $1/20 \times \text{FW}$ with CaCl_2).

We also observed a salinity-dependent expression of *pth1*. The expression level of *pth1* increased in $1/20 \times \text{FW}$, but decreased in $100 \times \text{FW}$ (Fig. 4). Importantly, *pth1* expression returned to standard levels in $1/20 \times \text{FW}$ supplemented with CaCl_2 (Fig. 7, $1/20 \times \text{FW}$ with CaCl_2), indicating that *pth1* expression depends on the external Ca^{2+} level. *pth1* regulation in zebrafish larvae is similar to regulation in mammals where *pth* is induced by a decrease in Ca^{2+} level in the plasma (Jüppner *et al.* 2000). In zebrafish, which do not have parathyroid glands, *pth1* is primarily expressed in the CNS (Hogan *et al.* 2005). In preliminary experiments, we observed an increase in *pth1* expression in the CNS in response to lower Ca^{2+} levels (K Hoshijima, unpublished observations). An interesting question for further investigation is whether the regulation of *pth1* expression in the CNS is conserved with regulation in the parathyroid glands in mammals.

This study demonstrates that the zebrafish endocrine system can respond to environmental salinity. The small size (~ 5 mm length at 7 dpf) of zebrafish larvae would restrict biochemical or physiological analyses; however, accessibility to genetic manipulation including loss of function analyses with antisense oligonucleotides should be a great advantage for the functional study of endocrine genes (McGonnell & Fowkes 2006). A comprehensive profiling of gene expression would also be useful for discovery of genes involved in the endocrine system. In fact, we have completed a preliminary examination of gene expression profiles under different salinity conditions, and have successfully identified hundreds of genes, whose expression is regulated by the external salinity (K, Hoshijima, unpublished observations). Taken together with the observations presented here, these comparative studies with zebrafish larvae should significantly contribute to our understanding of the regulatory mechanism of the endocrine system in vertebrates.

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