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

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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 Ca²⁺ 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.

Journal of Endocrinology (2007) 193, 481–491

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. 1953, Bolez-Feyset 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 (Abbnink & Flik 2006, Guerreiro et al. 2007), and the hypercalcemic function of PTHrP has been shown in sea bream (Abbnink et al. 2006, 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 per 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 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>
<thead>
<tr>
<th>Ion/osmolyte (μM)</th>
<th>Osmolarity (mOsmol/l)</th>
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<tbody>
<tr>
<td>Na(^{+})</td>
<td>984</td>
</tr>
<tr>
<td>K(^{+})</td>
<td>280</td>
</tr>
<tr>
<td>Ca(^{2+})</td>
<td>280</td>
</tr>
<tr>
<td>Cl(^{-})</td>
<td>804</td>
</tr>
<tr>
<td>SO(_4^{2-})</td>
<td>41.6</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>2-08</td>
</tr>
<tr>
<td>1× FW</td>
<td>–</td>
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</table>

Table 1 The concentration of the major ions and osmolarity in conditioned freshwater
concentrated medium (Table 1). For long-term acclimation to 1/20× FW and 100× FW, 10–25 fertilized embryos reared in 1× FW were dechorionated at 24 hours post-fertilization (hpf), rinsed with 1/20× FW or 100× FW, and cultured in 50 ml 1/20× FW or 100× FW in 100 mm Petri dish for 6 days respectively (Fig. 1A). For short-term exposure to 1/20× FW and 100× FW, 10–25 fertilized embryos reared in 1× FW were dechorionated at 24 hpf, rinsed with 1× FW and continuously cultured in 50 ml 1× FW in 100 mm petri dish. At 6 days post-fertilization (dpf), the larvae were rinsed with 1/20× FW or 100× FW and cultured in 50 ml 1/20× FW or 100× 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× FW or 100× FW as described above, and were transferred back to 1× FW on 6 dpf (=5 days post-acclimation) and cultured for 24 h. In a series of acclimation tests in 1/20× 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× FW. As a control, dechorionated embryos were continuously cultured in 1× 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× FW (shaded box): standard freshwater medium, 1/20× FW (open box): 20-fold diluted freshwater medium, 100× 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× FW medium (hatched box). (B) Viability of zebrafish larvae cultured in 1/20× FW or 100× 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× FW or 100× 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 µl 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 µl reaction volume using Superscript III First-Strand System (Invitrogen) and finally diluted in 40 µl diethyl pyrocarbonate-treated water. Real-time PCR was set up using 2.5 µl cDNA, 12.5 µl 2×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 µl. 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 (Ct) 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×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× 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/Napthol 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×FW) was the medium used for the routine culture of zebrafish embryos/larvae. We also prepared 1/20× FW and 10× FW, and transferred the larvae to these media at different developmental points (Fig. 1A). When larvae were transferred from 1× FW to 1/20× FW at 24 hpf and

Table 2 Primers used for quantitative PCR

<table>
<thead>
<tr>
<th>Target gene</th>
<th>GenBank accession no.</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Product size (bp)</th>
<th>Amplification efficiency (%)</th>
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<tbody>
<tr>
<td>anp</td>
<td>NM_198800</td>
<td>AGCAACATGGCCAAGCTCAAG</td>
<td>CAGGGGCTCCCTCAAACCTGC</td>
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<td>renin</td>
<td>NM_212860</td>
<td>TGAACTGGCAGAAGGAGGTTA</td>
<td>GCAACAGTGCCGAAACACCT</td>
<td>63</td>
<td>85-4</td>
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<tr>
<td>prolactin</td>
<td>NM_181437</td>
<td>GGCCTGGACACGCTGTA</td>
<td>ACCGGGAGATGGCACGTTGTG</td>
<td>61</td>
<td>98-8</td>
</tr>
<tr>
<td>gh</td>
<td>NM_001020492</td>
<td>CACTGAGAAACTGGTGGACCTG</td>
<td>TCCCTGTAGTAGCAGCCTG</td>
<td>52</td>
<td>86-5</td>
</tr>
<tr>
<td>pth1</td>
<td>NM_212950</td>
<td>AGAACCGCAACGGGACGTA</td>
<td>TGAACACCGAGATTATGTCAGCAG</td>
<td>62</td>
<td>86-6</td>
</tr>
<tr>
<td>rpl9</td>
<td>NM_001003861</td>
<td>TCCGATGGACGCGAAGGAG</td>
<td>TCGTCTTTTGGACGCAAGACA</td>
<td>52</td>
<td>87-8</td>
</tr>
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</table>
cultured for 6 days, viability was similar to that of control larvae (>95% viability), which were continuously cultured in 1×FW for 7 days (Fig. 1B). However, we observed that most of the larvae cultured in 1/20×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×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×FW to 100×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×FW and 100×FW (Fig. 1A). The zebrafish larvae that were transferred from 1×FW to 1/20×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×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×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×FW for 5 days (24 hpf to 6 dpf), zebrafish larvae were transferred back to 1×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×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×FW is reversible and quickly recovered under standard salinity conditions.

Figure 2 Morphology of zebrafish larvae cultured in different salinity conditions. (A–D) Morphology of 7 dpf zebrafish larvae cultured in 1×FW (A), acclimated in 1/20×FW (long term) (B), acclimated in 100×FW (long term) (C), and acclimated in 1/20×FW for 5 days and then in 1×FW for 24 h (readaptation) (D). (E–J) Morphology of 7-dpf zebrafish larvae cultured in 1/20×FW+sorbitol (E), in 1/20×FW+NaCl (F), in 1/20×FW+KCl (G), in 1/20×FW+NaCl, KCl (H), in 1/20×FW+CaCl2 (I), and in 1/20×FW+NaCl, KCl, CaCl2 (J). Arrowheads indicate the swimbladder (missing in B, E, G, and H). Scale bars, 1 mm.

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Table 3  PO2/PCO2 in conditioned freshwater. Mean and s.e.m. values were obtained by triplicate measurements.

<table>
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<tr>
<th>Medium</th>
<th>In stocka</th>
<th>In cultured dishb</th>
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<tbody>
<tr>
<td>1× FW</td>
<td>25·31±0·13</td>
<td>27·28±2·25</td>
</tr>
<tr>
<td>1/20× FW</td>
<td>25·35±0·44</td>
<td>24·49±0·20</td>
</tr>
<tr>
<td>100× FW</td>
<td>25·91±0·56</td>
<td>23·55±0·21</td>
</tr>
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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.
Finally, we also analyzed expression level of these genes after readaptation to $1 \times$ FW from $1/20 \times$ FW and $100 \times$ FW. Following readaptation to $1 \times$ FW for 24 h after 5 days culture in $1/20 \times$ FW, the expression of anp, renin, prolactin, gh, and pth1 was reduced to levels seen in standard conditions (Fig. 5). The expression level of prolactin and gh after readaptation from $1/20 \times$ 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 $1 \times$ FW for 24 h after 5 days culture in $100 \times$ 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 obsolete 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 ($24 \text{hpf-7 dpf}$) in $1/20 \times$ FW with sorbitol, in which the osmolarity was equal to $1 \times$ FW but the ionic strength remained $1/20 \times$, 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 $1/20 \times$ FW with NaCl, in which Na$^+$ was added so as to be equal to $1 \times$ (Cl$^-$ and osmolarity was also recovered to $0.85 \times$), while other ions remained at $1/20 \times$ concentration, the larvae exhibited severe morphological defects accompanied by a reduction of viability. When cultured in $1/20 \times$ 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 $1/20 \times$ 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 $1/20 \times$ FW with NaCl neutralized the toxicity caused by elevated Na$^+$. When larvae were cultured for 6 days in $1/20 \times$ FW with NaCl and KCl, in which both Na$^+$ and K$^+$ were raised to $1 \times$ 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 $1/20 \times$ FW with CaCl$_2$, in which Ca$^{2+}$ was equal to $1 \times$ but other ions remained $1/20 \times$ concentration.
significant differences in expression levels for any gene between 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.

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
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. 1/20FW, hypervolemia and hyponatremia would be more extreme. We speculate that anp expression in 1/20FW 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, Manzon 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.

**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≥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).
Alternatively, more GH may be required for larval growth including bone formation when Ca$^{2+}$ availability is limited in 1/20× FW, leading to the higher expression level. Supporting this hypothesis, the expression level of gh decreased upon addition of Ca$^{2+}$ to 1/20× FW (Fig. 7, 1/20× FW with CaCl$_2$).

We also observed a salinity-dependent expression of pth1. The expression level of pth1 increased in 1/20× FW, but decreased in 100× FW (Fig. 4). Importantly, pth1 expression returned to standard levels in 1/20× FW supplemented with CaCl$_2$ (Fig. 7, 1/20× 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.

Acknowledgements

We thank Dr Hidekazu Fukuda for technical assistant and Dr Lisa M Goering for copyediting the manuscript. This work was supported by Grants-in-Aid for Scientific Research (14104002, 17570003, and 18059010) from the Ministry of Education, Culture, Sport, Science and Technology of Japan (MEXT) and the 21st Century Center of Excellence (COE) Program of MEXT. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received in final form 28 March 2007

Accepted 2 April 2007

Made available online as an Accepted Preprint 3 April 2007