Zebrafish pituitary gene expression before and after sexual maturation

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Abstract

Sexual maturation and somatic growth cessation are associated with adolescent development, which is precisely controlled by interconnected neuroendocrine regulatory pathways in the endogenous endocrine system. The pituitary gland is one of the key regulators of the endocrine system. By analyzing the RNA sequencing (RNA-seq) transcriptome before and after sexual maturation, in this study, we characterized the global gene expression patterns in zebrafish pituitaries at 45 and 90 days post-fertilization (dpf). A total of 15 043 annotated genes were expressed in the pituitary tissue, 3072 of which were differentially expressed with a greater than or equal to twofold change between pituitaries at 45 and 90 dpf. In the pituitary transcriptome, the most abundant transcript was *gh*. The expression levels of *gh* remained high even after sexual maturation at 90 dpf. Among the eight major pituitary hormone genes, *lhb* was the only gene that exhibited a significant change in its expression levels between 45 and 90 dpf. Significant changes in the pituitary transcripts included genes involved in the regulation of immune responses, bone metabolism, and hormone secretion processes during the juvenile–sexual maturity transition. Real-time quantitative PCR analysis was carried out to verify the RNA-seq transcriptome results and demonstrated that the expression patterns of the eight major pituitary hormone genes did not exhibit a significant gender difference at 90 dpf. For the first time, we report the quantitative global gene expression patterns at the juvenile and sexual maturity stages. These expression patterns may account for the dynamic neuroendocrine regulation observed in body metabolism.

Key Words
- zebrafish
- pituitary
- RNA-seq transcriptome
- endocrine signal
- adolescent transition

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Introduction

The pituitary gland is a central endocrine gland that regulates the function of other organs and responds to the hypothalamic centers involved in processing external and internal stimuli. The pituitary gland of teleost fish is unique among that of vertebrates in that it lacks a hypothalamic–pituitary portal system. The teleost anterior pituitary is directly innervated by nerve fibers from the hypothalamus. This represents the magnificent manifestation of all the fundamental physiological processes directly linked to the survival of fish across a great range of environments, including their somatic growth, gonadal maturity, reproductive functions, appetite, and stress responses (Trudeau *et al.* 2000, Wong *et al.* 2006, Kurata *et al.* 2012).

The pituitary gland of teleost fish synthesizes and secretes various peptide hormones, including growth hormone (Gh), proopiomelanocortin (Pomc), prolactin (Prl), somatolactin (Smtl), thyroid-stimulating hormone

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(Tsh), luteinizing hormone (Lh), and follicle-stimulating hormone (Fsh). Each of the pituitary hormones is released into the bloodstream and acts on different target organs to strictly regulate a variety of biological activities, thereby maintaining physiological homeostasis, growth, and reproduction (Lohr & Hammerschmidt 2011, Perez-Castro *et al.* 2012). Many of these peptide hormones are synthesized as inactive precursors, which when converted to their mature forms by proteolytic enzymes generate a large diversity of bioactive proteins and peptides. A family of the proprotein convertases (PCs) actively participates in the generation of such molecular diversity (Morash *et al.* 2009). Moreover, the expression, synthesis, and secretion of these hormones are regulated by neuroendocrine feedback systems (Canosa *et al.* 2007, Martyniuk *et al.* 2012). Genes expressed in the pituitary gland have also been identified in some gene profiling studies carried out previously with microarray or RT-PCR analyses (Kappeler *et al.* 2003, Zhang *et al.* 2009). It is likely that a large number of genes, not just the classical pituitary hormones, are essential for the endocrine homeostatic systems of the pituitary gland (Perez-Castro *et al.* 2012). However, due to the disadvantages and limitations of the techniques employed in previous transcriptome analyses, global gene profiles of the animal pituitary typically lack comprehensiveness and accuracy.

Zebrafish (*Danio rerio*) is one of the commonly used vertebrate model organisms in developmental biology with high-quality genetic information. Despite the vast emergence of available information, very little work has been carried out to evaluate zebrafish as a model species for somatic growth studies, including applications to aquaculture (Lohr & Hammerschmidt 2011). Zebrafish is phylogenetically closer to the farmed cyprinid fish species than any other vertebrate model, which makes it a useful organism to study the gene functions of aquaculture fish. All juvenile zebrafish develop an ovary-like structure (juvenile ovary), which will either develop into a definite ovary in females or transform into a testis in males around 45 days post-fertilization (dpf; Chen & Ge 2013). It takes ~3 months to reach sexual maturity (Clelland & Peng 2009). Puberty is defined as the period that marks the transition from sexual immaturity to maturity. It has been suggested that the onset of ovary maturation in female zebrafish occurs at around 45 dpf (Chen & Ge 2013). Under standard laboratory conditions, zebrafish normally exhibit a growth burst stage within 9–51 dpf, coinciding with the critical stage of their sex differentiation. Their growth rates usually significantly decrease after 65 dpf (Gomez-Requeni *et al.* 2010). Numerous gene expression profiling studies have been carried out using different organs or tissues, but very few studies have addressed questions on the developmental changes in pituitary gene expression due to maturation and somatic growth processing in zebrafish.

Identification of actively transcribed genes is fundamental to the understanding of the function, molecular events, and physiology of specific tissues. Somatic growth is directly regulated by the endocrine somatotropic axis, where Gh functions as a major secreted component within the pituitary gland (Lohr & Hammerschmidt 2011, Perez-Castro *et al.* 2012). To understand the expression and production of hormones by the pituitary gland, orchestrated by changes in physiological homeostasis during the growth burst stage and the adult slow somatic growth stage, in this study, we dissected pituitaries of zebrafish at 45 and 90 dpf and carried out a global transcriptome analysis on them using the RNA-seq technique. The results of this analysis provide a global view of the transcriptional features in the zebrafish pituitary in correlation with the transition from the juvenile stage to the sexual maturity stage. Thus, the present study provides an increased mechanistic understanding of the gene networks that are involved in the endocrine somatotropic axis in the teleost pituitary during development.

Materials and methods

Animals and sampling

The WT AB strain of zebrafish was inbred for 8 years in our laboratory following the procedure described previously (Westerfield 2000). Zebrafish were maintained in dechlorinated tap water at 28.5 °C in a 14h light:10h darkness photoperiod and fed three times a day with brine shrimp. All the animal protocols were approved by the Institute Research Board (IRB) of the Institute of Hydrobiology, Chinese Academy of Sciences (no. IHB2012005). Under these conditions, most of the zebrafish reached sexual maturity in our laboratory. Approximately 300 pituitaries were obtained from juvenile zebrafish at 45 dpf, as described previously (Toro *et al.* 2009). An additional 200 pituitaries were dissected from adult zebrafish at 90 dpf. All the dissected pituitaries were transferred into RNAlater solution (Ambion, Thermo Fisher Scientific, Waltham, MA, USA) immediately after dissection. Pituitaries were collected from both male and female zebrafish. Owing to the labor-intensive work and sequencing costs, samples were not prepared for different replicates before mRNA-seq

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library construction. However, the use of a large number of pituitaries (~200–300 per stage) enabled us to assay the average expression profile of each gene across different biological individuals.

In addition, 60 pituitaries from juvenile zebrafish at 45 dpf, 30 pituitaries from female adult zebrafish at 90 dpf, and 30 pituitaries from male adult zebrafish at 90 dpf were collected as samples for quantitative real-time PCR.

RNA isolation and preparation for RNA-seq

Total RNA was isolated from pituitary tissue using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The sample was then treated with RNase-free DNase I (Promega). RNA concentrations were determined using Qubit 2.0 (Life Technologies, Thermo Fisher Scientific), and RNA size and integrity were ensured by analyzing the samples on a 1.5% (w/v) agarose gel using the Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA). To avoid amplification of the RNA samples, the amount of total RNA for each sample should be more than 4.0 μg with the RNA integrity number (RIN) >8.0. Poly-A-containing mRNA was further isolated using the PolyATract mRNA Isolation System (Promega) from each of the total RNA samples. RNA fragments were converted into first-strand cDNA using reverse transcriptase and random primers, followed by second-strand cDNA synthesis using DNA polymerase I and RNase H. After the end repair process and adapter ligation step, the products were purified and amplified using PCR to generate the final cDNA libraries.

Transcriptome analysis

A total of 95 base sequences were obtained by sequencing using Illumina HiSeq 2000. The sequencing-received raw image data were transformed by base culling into sequence data. Before mapping the reads to the reference database, all the sequences were filtered to remove adaptor sequences and low-quality sequences. The remaining reads were aligned to the zebrafish genome using SOAPaligner/soap2, allowing up to two base mismatches. All the reads were aligned against the zebrafish genome assembly version 9 (Zv9; http://www.sanger.ac.uk). The normalized gene expression level was separately calculated as reads per kilobase of mRNA length per millions of mapped reads (RPKM) for each library. This facilitated the comparison of the transcript levels between the samples. The cutoff value for determining the gene transcriptional activity was determined based on a 95% CI for all the RPKM values for each gene. Gene ontology (GO) functional enrichment analysis was carried out using Blast2GO (version 2.3.5; http://www.blast2go.org/). KEGG pathway analysis was carried out using the Cytoscape Software (version 2.6.2; http://www.cytoscape.org/) with the ClueGO plugin (http://www.ici.upmc.fr/cluego/cluegoDownload.shtml).

RT-PCR and quantitative real-time PCR

To carry out further analyses of sexually dimorphic gene expression in the pituitary gland, the expression levels of mRNAs of six major hormones were determined in the pituitaries of juveniles, adult males, and adult females using RT-PCR. Briefly, total RNA was converted into first-strand cDNA using the RevertAid First Strand cDNA Synthesis Kit (Fermentas, Thermo Fisher Scientific) with oligo-dT primers. PCRs were then carried out in a 20 μl reaction mixture containing 10 μl of SYBR Green Real-time PCR Master Mix Plus (Toyobo, Osaka, Japan), 0.25 μm each of forward and reverse primers, and 1 μl of cDNA sample. The relative abundance of mRNAs was calculated using the comparative cycle of threshold method with β-actin1, β-actin2, and ribosomal protein L13a (*rpl13a*) mRNAs as triple internal standards. These measurements were performed in triplicate and had a coefficient of variation <5%. First, the expression levels of the target genes were normalized to those of the three internal controls, β-actin1, β-actin2, and *rpl13a*, within each sample. Then, to compare the relative expression levels of the major pituitary hormones in the pituitary samples obtained from adult fish with those in the samples obtained from juvenile fish, target gene expression levels at the juvenile stage were set to 'onefold'. Pairwise comparisons between pituitary samples obtained from adult fish and those obtained from juvenile fish were recorded as fold changes (mean ± S.E.). The primers used are listed in Table 1. The levels of gene expression were measured with three independent biological replicates for each condition, and each biological replicate sample was run with three technical replicates.

Results

RNA-seq of the zebrafish pituitary transcriptome

For RNA-seq of the zebrafish pituitary transcriptome, 300 pituitaries were dissected from zebrafish at 45 dpf (P45 group) and 200 pituitaries from zebrafish at 90 dpf (P90 group). The total RNA yields for each group were 4.35 μg (P45) and 4.52 μg (P90), with RIN values of 8.6 and 8.2 respectively. After generation of cDNA samples for the P45

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Table 1 Primers used in quantitative real-time RT-PCR

Genes (symbol)	**Forward primer sequence**	**Reverse primer sequence**
Thyroid-stimulating hormone-β (*tshb*)	5′-CTGTCAACACCACCATCTGC-3′	5′-GTGCATCCCCTCTGAACAAT-3′
Proopiomelanocortin a (*pomca*)	5′-AGCTCAGTGTTGGGAAAACG-3′	5′-GGTAGACGGGGGTTTCATCT-3′
Prolactin (*prl*)	5′-TTGGAAGGGGAATGATGCCG-3′	5′-GACTGGACGCCTCAGAAGAG-3′
Growth hormone (*gh*)	5′-CCTCTGTCGTTCTGCAACTC-3′	5′-ACTCCCAGGATTCAATGAGG-3′
Luteinizing hormone (*lhb*)	5′-GCAGAGACACTTACAACAGCC-3′	5′-AAAACCAAGCTCTGGAGCAGCC-3′
Follicle-stimulating hormone-β (*fshb*)	5′-GATGCGTGTGCTTGTTCTGG-3′	5′-ACTCGATCCATTGTCCAGCAT-3′
β-Actin1	5′-CGAGCAGGAGATGGGAACC-3′	5′-CAACGGAAACGATCATTGC-3′
β-Actin2	5′-CGTGCTGTCTTCCCATCCA-3′	5′-TCACCAACGTAGCTGTCTTTCTG-3′
Ribosomal protein L13a (*rpl13a*)	5′-TCTGGAGGACTGTAAGAGGTATGC-3′	5′-AGACGCACAATCTTGAGAGCAG-3′

and P90 groups, a total of ∼99 million, 95-bp double-end reads were generated. Among the raw reads, ∼38 million (P45 sample) and 36 million (P90 sample) reads were mapped to the zebrafish reference sequence. A cutoff value of 1.0 RPKM was established for either of the pituitary samples to evaluate the relative abundance levels of transcripts that were considered for further analysis. In both pituitary samples, a total of 15 043 annotated genes were present at above 1 RPKM. Among them, 12 579 genes were expressed in both the P45 and P90 samples. In addition, 1909 genes were expressed in the pituitary gland only at 45 dpf and 555 genes only at 90 dpf (Fig. 1). The annotation of these expressed genes was achieved using BLASTN similarity searches against the Ensembl zebrafish RefSeq mRNA database (Version danRer 7, Supplementary Tables 1 and 2, see section on supplementary data given at the end of this article).

Identification of differentially expressed genes in the pituitaries at the juvenile and sexual maturity stages

To identify the differentially expressed genes, the transcriptome data of zebrafish pituitaries were analyzed using previously described methods (Audic & Claverie 1997). The cutoff value for comparison was established at 1.0 RPKM for the transcripts in either the P45 or P90 sample. The criteria of a twofold or greater change in expression and P value <0.05 (cutoff at a 1% false discovery rate) were chosen to determine significant differences in expression. Using these criteria, a total of 3071 genes were found to be significantly differentially expressed, including 586 upregulated genes and 2485 downregulated genes in the P90 sample (Supplementary Table 3, see section on supplementary data given at the end of this article). The results of the GO analysis (by GOView.html) and KEGG pathway analysis indicated that the differentially expressed genes were highly related to focal adhesion, extracellular matrix–receptor interaction, and protein binding (Supplementary Fig. 1).

Transcriptional levels of the major zebrafish pituitary hormones before and after sexual maturity

The sum of the RPKM values of the ten transcripts with the highest RPKM in the pituitary samples comprised 47.34 or 51.73% of the sum of all transcripts in the P45 or P90 sample respectively. Among the top ten most abundant transcripts, *gh* was the most highly expressed one in both the P45 and P90 samples. The RPKM of the *gh* gene alone in the P45 and P90 samples contributed to ∼32.96 and 26.80% of the sum of the RPKM values of the total transcripts in the P45 and P90 samples respectively. However, the expression levels of *gh* only decreased by ∼19.7% in the pituitary gland from 45 to 90 dpf (Table 2). In addition to those that were among the top ten transcripts, the other major pituitary hormone transcripts, including glycoprotein hormones α-polypeptide 1 variant b (*cga-1b*), Smtlβ (*smtlb*), TSHβ-subunit (*tshb*), and FSHβ-polypeptide (*fshb*), are summarized in Table 2. Among these, only the expression levels of *lhb* ($2^{2.38}$-fold) and *cga-1b* ($2^{1.36}$-fold) were significantly higher at 90 than at 45 dpf (Fig. 2, Table 2, and Supplementary Table 3).

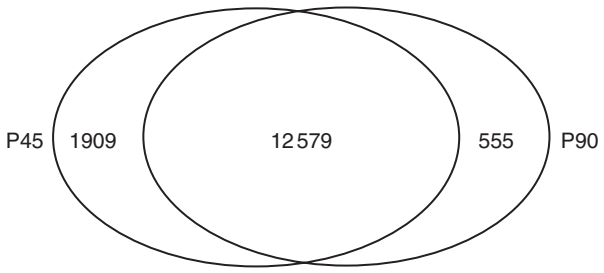

Figure 1
Comparison of the differential expression of identified genes in the pituitaries at 45 and 90 dpf. The number in the overlapped region represents the annotated genes that were significantly expressed (>1.0 RPKM) in both samples. The numbers in the nonoverlapped regions represent the annotated genes that were significantly expressed (>1.0 RPKM) in only one of the samples.

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Table 2 Comparison of the major categories of transcripts in the juvenile (P45) and adult (P90) zebrafish pituitary transcriptomes

Ensembl transcript ID	Associated gene name	Description	RPKM in the P45 sample (rank in the abundance; the percentage of the transcriptome)	RPKM in the P90 sample (rank in the abundance; percentage of the transcriptome)	RPKM in the P90 sample/RPKM in the P45 sample
Major pituitary hormones			306 656.88 (40.34%)	331 344.34 (44.08%)	1.08
ENSDAR-T00000055675	*gh1*	Growth hormone 1	250 615.76 (no. 1; 32.96%)	201 441.28 (no. 1; 26.80%)	0.80
ENSDAR-T00000063333	*pomca*	Proopiomelanocortin a	18 882.67 (no. 3; 2.48%)	24 983.64 (no. 3; 3.32%)	1.32
ENSDAR-T00000053888	*cga-1a*	Glycoprotein hormones, α-polypeptide-1a	12 284.88 (no. 5; 1.62%)	21 739.49 (no. 4; 2.89%)	1.77
ENSDAR-T00000051787	*lhb*	Luteinizing hormone, β-polypeptide	10 462.52 (no. 7; 1.38%)	54 416.38 (no. 2; 7.24%)	5.20
ENSDAR-T00000055299	*prl*	Prolactin	6881.07 (no. 9; 0.91%)	13 116.26 (no. 9; 1.74%)	1.91
ENSDAR-T00000059245	*cga-1b*	Glycoprotein hormones, α-polypeptide 1b	5910.22 (no. 11; 0.78%)	13 209.76 (no. 8; 1.76%)	2.24
ENSDAR-T00000129647	*smtlb*	Somatolactin-β	969.28 (no. 85; 0.13%)	1790.67 (no. 45; 0.24%)	1.85
ENSDAR-T00000050000	*tshb*	Thyroid-stimulating hormone, β-subunit	336.80 (no. 158; 0.04%)	244.39 (no. 176; 0.03%)	0.73
ENSDAR-T00000011581	*fshb*	Follicle-stimulating hormone, β-polypeptide	313.68 (no. 168; 0.04%)	402.47 (no. 122; 0.05%)	1.28
Other major secreted factors[a]			12 992.25 (1.71%)	20 621.30 (2.74%)	1.59
Signal receptors			2017.24 (0.27%)	2144.93 (0.29%)	1.06
Proprotein convertase (subtilisin/kexin type)			658.54 (0.09%)	1064.82 (0.14%)	1.62
Ribosomal proteins			98 989.71 (13.02%)	94 696.11 (12.60%)	0.96
Mitochondrial proteins			88 170.90 (11.60%)	86 635.99 (11.53%)	0.98
Others			250 787.16 (32.99%)	215 189.07 (28.63%)	0.86
Total			760 272.68	751 696.23	0.99

Reads per kilobase of mRNA length per millions of mapped reads.
[a]The major pituitary secreted factors other than the pituitary hormones with the values of RPKM >100 in either the juvenile or adult pituitary transcriptome. These transcripts are summarized in Table 3.

The relative expression levels of ribosomal proteins and mitochondrial proteins in the total transcript pools remained nearly unchanged in the P45 (13.02 and 11.60%) and P90 (12.60 and 11.53%) samples (Table 2). Similar trends were also observed in the relative levels of the total major pituitary hormone transcription in the P45 (40.34%) and P90 (44.08%) samples (Table 2). However, the relative percentages of *lhb* and *cga-1b* transcripts in the pool of the major pituitary hormone transcripts were significantly higher at 90 than at 45 dpf (Table 2).

Identification of additional differentially expressed secreted signaling molecules in the pituitaries at juvenile and adult stages

The pituitary gland produces and releases many endocrine hormones, growth factors, and cytokines. Hormone secretion by the pituitary gland is also regulated by secreted factors via bloodstream input (Perez-Castro *et al.* 2012). We next identified additional secreted molecules or receptors that were differentially expressed in the pituitary gland.

The expression levels of all the highly abundant secreted protein genes (>100.0 RPKM for those in either the P45 or P90 sample) are summarized in Table 3. Among the major secreted molecules and the molecules involved in the processing of protein secretion, only secretogranin III (*scg3*), thymosin-β 15a (*tmsb15a*), neuromedin S (*nms*), ependymin (*epd*), secreted acidic cysteine-rich glycoprotein (*sparc*), secretory calcium-binding phosphoprotein 7 (*scpp7*), secretogranin V (*scg5/7B2*), and granulin 1 (*grn1*), in addition to *lhb* and *cga-1b*, were significantly differentially expressed in the pituitary gland from 45 to 90 dpf. Among these genes, the expression levels of *scg3*, *nms*, *scg5*, and *grn1* were significantly increased, while those of *tmsb15a*, *epd*, *sparc*, and *scpp7* decreased significantly from 45 to 90 dpf (Table 3).

Some transcription factors (TFs), such as Rathke's pouch homeobox (Rpx), pituitary homeobox 3 (Pitx3), LIM-homeobox-3 (Lhx3), Lhx4, T-box transcription factor

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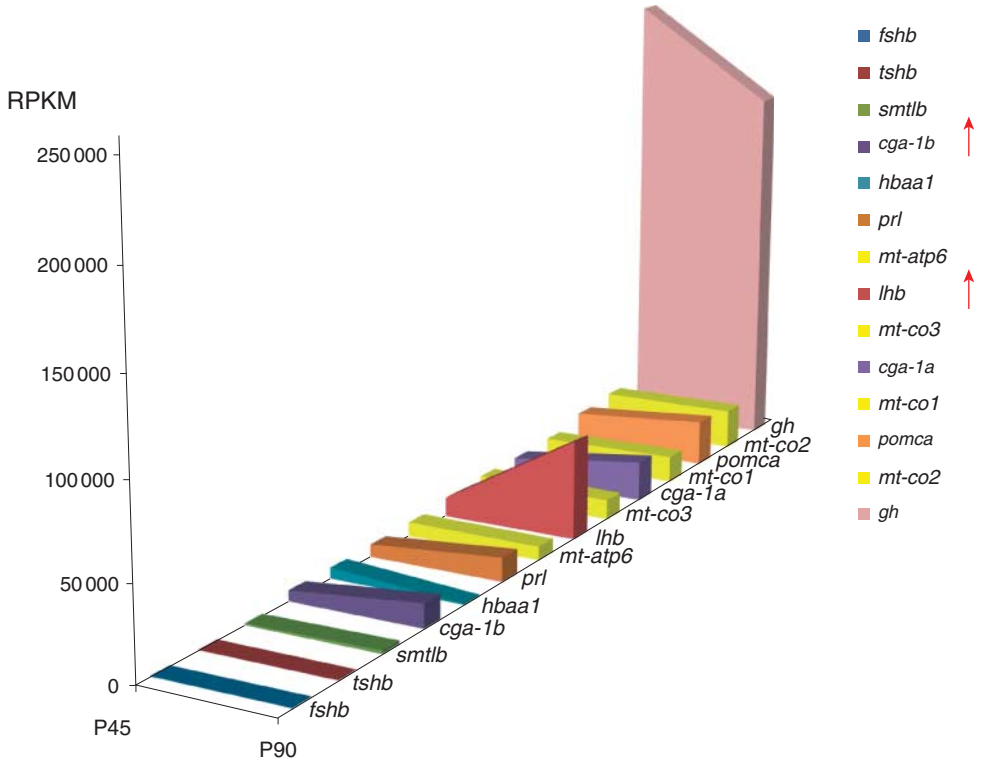

Figure 2
Expression profiles of the ten transcripts with the highest RPKM and major pituitary hormone transcripts during the transition from the juvenile to the sexual maturity stage. The ten pituitary transcripts with the highest RPKM in the pituitary gland at the juvenile stage were growth hormone (*gh*), mitochondrial cytochrome *c* oxidase II (*mt-coII*), proopiomelanocortin a (*pomc*), mitochondrial cytochrome *c* oxidase I (*mt-coI*), glycoprotein hormones, α-polypeptide 1 variant a (*cga-1a*), mitochondrial cytochrome *c* oxidase III (*mt-coIII*), luteinizing hormone β-polypeptide (*lhb*), mitochondrial ATP synthase 6 (*mt-atp6*), prolactin (*prl*), and hemoglobin-α adult-1 (*hbaa1*). The other major pituitary hormone transcripts included glycoprotein hormones, α-polypeptide 1 variant b (*cga-1b*), somatolactin-β (*smtlb*), thyroid-stimulating hormone, β-subunit (*tshb*), and follicle-stimulating hormone, β-*polypeptide* (*fshb*). The only two significant changes between the transcriptional levels at the two stages are labeled with a red arrow (↑).

(Tbx19), Islet1 (Isl1), POU domain, class 1, transcription factor 1 (Pou1f1), prophet of Pou1f1 (Prop1), GATA-binding protein 2 (GATA2), Sry-box-containing gene 2 (Sox2), and Sox3, regulate the promotion and determination of the pituitary cell lineages. These TFs have also been suggested to be the key regulators of the modulation of pituitary hormone gene expression (Cohen & Radovick 2002, Pfaffle & Klammt 2011). However, the expression patterns of these TFs did not exhibit significant changes between 45 and 90 dpf (Supplementary Table 3). Among the critical signaling molecules identified in the early developmental processing of the pituitary gland, only the expression levels of the steroidogenic factor 1 (*sf1* or *nr5a1*) changed significantly between 45 and 90 dpf (Cohen & Radovick 2002, Pfaffle & Klammt 2011; Table 4).

Our analyses revealed the presence of a number of PC subtilisin/kexin type (*pcsk*) transcripts, including *pcsk1*, *pcsk2*, *pcsk5*, *pcsk5b*, *pcsk6*, and *pcsk7*, in the pituitary tissue samples at both 45 and 90 dpf (Supplementary Tables 1 and 2). Among these *pcsk* transcripts, *pcsk2* was highly abundant (Supplementary Tables 1 and 2), and only *pcsk5* exhibited significantly different expression levels between 45 and 90 dpf (Supplementary Table 3). Moreover, highly abundant transcripts of genes involved in protein secretion or PC activity regulation, such as *scg3*, *scg5*, and *pcsk1* inhibitor, like (*pcsk1nl* or *proSAAS*), were found in the pituitary tissue samples. Scg3 is a secretory protein involved in protein secretion, although no clear biological function has been found in a mammalian animal model (Bartolomucci *et al*. 2011). Pcsk1n is a specific endogenous inhibitor of Pcsk1, which may inhibit the convertase-mediated processing of Pomc and proenkephalin (Kudo *et al*. 2009). Scg5 is another secretory protein involved in protein secretion in a mammalian animal model. Scg5 functions as a chaperone to regulate PC2 in mouse and is involved in post-translational endoproteolytic Pomc

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Table 3 Major pituitary secreted factors other than the pituitary hormones[a]

Ensembl transcript ID	Associated gene name	Description	RPKM in the P45 sample	RPKM in the P90 sample	Log2 ratio (P90:P45)	Up- or down-regulation (P90/P45)	*P* value	FDR
ENSDART00000122930	*scg3*	Secretogranin III	5298.91	13 280.88	1.33	Up	0	0
ENSDART00000004679	*Icn*	Ictacalcin	1680.29	991.80				
ENSDART00000104475	*Tmsb15a*	Thymosin-β 15a	1017.21	454.17	−1.16	Down	0	0
ENSDART00000111194	*Nms*	Neuromedin S	984.86	2493.64	1.34	Up	0	0
ENSDART00000049895	*Epd*	Ependymin	635.93	30.73	−4.37	Down	0	0
ENSDART00000111160	*ptmab*	Prothymosin, αb	629.92	326.90				
ENSDART00000003653	*scg2a*	Secretogranin II (chromogranin C), a	514.85	722.22				
ENSDART00000145282	*Sepp1a*	Selenoprotein P, plasma, 1a	340.95	210.34				
ENSDART00000039660	*sparc*	Secreted acidic cysteine-rich glycoprotein	322.08	80.30	−2.00	Down	0	0
ENSDART00000017424	*ptmaa*	Prothymosin, αa	286.87	319.39				
ENSDART00000109283	*scpp7*	Secretory calcium-binding phosphoprotein 7	207.96	3.46	−5.91	Down	0	0
ENSDART00000112579	*scg2b*	Secretogranin II (chromogranin C), b	196.83	178.44				
ENSDART00000045249	*scg5*	Secretogranin V	194.43	514.78	1.40	Up	0	0
ENSDART00000128734	*adm2*	Adrenomedullin 2	192.34	215.98				
ENSDART00000081649	*scgn*	Secretagogin, EF-hand calcium-binding protein	161.37	119.41				
ENSDART00000109894	*olfm2*	Olfactomedin 2	160.84	216.73				
ENSDART00000003042	*mdkb*	Midkine-related growth factor b	130.52	75.10				
ENSDART00000051972	*grn1*	Granulin 1	36.09	387.03	3.42	Up	0	0

[a]The transcripts with values of RPKM >100 in either the juvenile or adult pituitary transcriptome.

processing (Bartolomucci *et al.* 2011). The expression levels of *Scg3* and *Scg5* were significantly higher at 90 dpf than at 45 dpf (Table 3). We found an additional 64 transcripts of various hormones, growth factors, cytokines, receptors, and molecules known to be involved in prohormone processing, such as gonadotropin-releasing hormone receptor 2 (*gnrhr2*), androgen receptor (*ar*), progesterone receptor (*pgr*), estrogen receptor 1 (*er1*), *er2b*, glutamate receptor, ionotropic, δ-1 (*grid1*), galanin prepropeptide (*gal*), dopamine receptor D1 (*drd1*), osteoglycin (*ogn*), insulin-like growth factors (*igfs*), interleukin 2 (*il2*), and platelet-derived growth factor-α (*pdgfa*), transforming growth factor-β (*tgfb*), and follistatin (*fst*), which were each significantly altered in the pituitary gland during the transition from the pubertal to the adolescent stage (Table 4).

Quantitative-PCR analysis of sexually dimorphic expression of the pituitary hormone genes

Pituitary tissue samples were collected from juvenile zebrafish (45 dpf, gender not distinguishable) and adult male or female zebrafish at 90 dpf. Total RNA was collected for experimental measurement of the expression patterns of the six major pituitary hormones. Real-time quantitative PCR (q-PCR) analysis was carried out with β-actin2, β-actin2, and *rpl13a* as triple internal controls within each sample. As shown in Fig. 3, only *lhb* exhibited clear increased expression levels in the pituitaries of both adult male and female fish. The results also revealed sexually dimorphic gene expression profiles of the eight major hormones in the pituitaries of adult fish, but not a significant gender-specific difference.

Discussion

Zebrafish grow rapidly from birth to ~50 dpf when most individuals begin to allocate part of their dietary energy intake to sexual maturation, which coincides with a decrease in growth rates (Gomez-Requeni *et al.* 2010). The pituitary gland is a key regulator of body homeostasis during development. A few studies have examined pituitary gene expression during early developmental stages (Brinkmeier *et al.* 2009, Toro *et al.* 2009). High-throughput RNA-seq has enhanced our ability to perform transcriptome analyses at a far higher resolution than

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Table 4 Other known molecules involved in endocrine regulation

Ensembl transcript ID	Associated gene name	Description	P1-RPKM	P2-RPKM	Log2 ratio (P2:P1)
ENSDART00000103811	*ctgfb*	Connective tissue growth factor b	5.048	0.255	−4.306
ENSDART00000106096	*drd1 (1 of 2)*	Dopamine receptor D1	6.705	19.448	1.536
ENSDART00000076082	*fetub*	Fetuin B	70.360	4.203	−4.065
ENSDART00000011701	*fgb*	Fibrinogen, B β-polypeptide	4.211	0.324	−3.699
ENSDART00000013024	*fgf20a*	Fibroblast growth factor 20a	1.681	4.224	1.329
ENSDART00000058745	*fgfbp3*	Fibroblast growth factor-binding protein 3	40.229	16.985	−1.244
ENSDART00000100286	*fgfr4*	Fibroblast growth factor receptor 4	9.164	20.285	1.146
ENSDART00000015046	*fstl1a*	Follistatin-like 1a	26.713	3.963	−2.753
ENSDART00000121456	*fstl1b*	Follistatin-like 1b	37.521	15.734	−1.254
ENSDART00000145434	*gpr52*	G-protein-coupled receptor 52	17.058	38.061	1.158
ENSDART00000130608	*gal*	Galanin prepropeptide	8.741	43.015	2.299
ENSDART00000124134	*gfral*	GDNF family receptor-α like	6.180	12.818	1.052
ENSDART00000064839	*grid1 (1 of 2)*	Glutamate receptor, ionotropic, δ1	3.754	7.936	1.080
ENSDART00000006795	*gnrhr2*	Gonadotropin-releasing hormone receptor 2	0.802	31.459	5.294
ENSDART00000109138	*hbegfa*	Heparin-binding EGF-like growth factor a	10.847	5.184	−1.065
ENSDART00000004034	*hpca*	Hippocalcin	12.374	3.481	−1.830
ENSDART00000145496	*icn2*	Ictacalcin 2	29.249	3.256	−3.167
ENSDART00000124779	*igflr1*	IGF-like family receptor 1	4.305	1.061	−2.021
ENSDART00000026576	*igfbp1a*	Insulin-like growth factor-binding protein 1a	99.758	19.404	−2.362
ENSDART00000074327	*igfbp2a*	Insulin-like growth factor-binding protein 2a	14.866	4.019	−1.887
ENSDART00000020400	*igfbp3*	Insulin-like growth factor-binding protein 3	31.176	14.632	−1.091
ENSDART00000054137	*igfbp5b*	Insulin-like growth factor-binding protein 5b	24.459	7.630	−1.681
ENSDART00000099732	*il2rga*	Interleukin 2 receptor, γa	5.544	0.393	−3.817
ENSDART00000091550	*iphn2 (1 of 2)*	Latrophilin 2	5.198	10.862	1.063
ENSDART00000106619	*nrn1*	Neuritin 1	6.311	0.865	−2.867
ENSDART00000149312	*ncalda*	Neurocalcin-δa	6.112	1.735	−1.817
ENSDART00000044733	*npbwr2 (2 of 2)*	Neuropeptides B/W receptor 2	48.266	226.364	2.230
ENSDART00000150863	*nrp2a*	Neuropilin 2a	8.120	3.742	−1.117
ENSDART00000028265	*nr5a1b*	Nuclear receptor subfamily 5, group A, member 1b	12.860	41.094	1.676
ENSDART00000064801	*ogn*	Osteoglycin	16.628	0.196	−6.407
ENSDART00000062885	*oxtl*	Oxytocin-like	15.160	40.172	1.406
ENSDART00000111730	*pappa2*	Pappalysin 2	2.730	12.138	2.152
ENSDART00000001320	*pdgfra*	Platelet-derived growth factor receptor-α	4.646	1.190	−1.965
ENSDART00000105088	*pdgfaa*	Platelet-derived growth factor-αa	6.283	2.896	−1.117
ENSDART00000007022	*pdgfrl*	Platelet-derived growth factor receptor-like	14.220	1.774	−3.003
ENSDART00000052206	*pgr*	Progesterone receptor	2.183	8.345	1.935
ENSDART00000060814	*paqr6*	Progestin and adipoQ receptor family member VI	12.541	2.798	−2.164
ENSDART00000076304	*pcna*	Proliferating cell nuclear antigen	122.835	54.746	−1.166
ENSDART00000111650	*pmch*	Pro-melanin-concentrating hormone	7.993	1.988	−2.008
ENSDART00000108958	*pcsk5*	Proprotein convertase subtilisin/kexin type 5	9.100	3.999	−1.186
ENSDART00000109588	*rbp4*	Retinol-binding protein 4, plasma	86.242	14.891	−2.534
ENSDART00000027000	*rho*	Rhodopsin	36.337	10.230	−1.829
ENSDART00000133225	*spp2*	Secreted phosphoprotein 2, 24 kDa	4.342	0.329	−3.721
ENSDART00000055687	*smtla*	Somatolactin-α	2.983	21.797	2.869
ENSDART00000034875	*sdf2*	Stromal cell-derived factor 2	12.376	5.968	−1.052
ENSDART00000003278	*tacr3 (2 of 3)*	Tachykinin receptor 3	1.757	4.818	1.456
ENSDART00000039832	*tgfbr2*	Transforming growth factor, β-receptor II	8.790	2.725	−1.690

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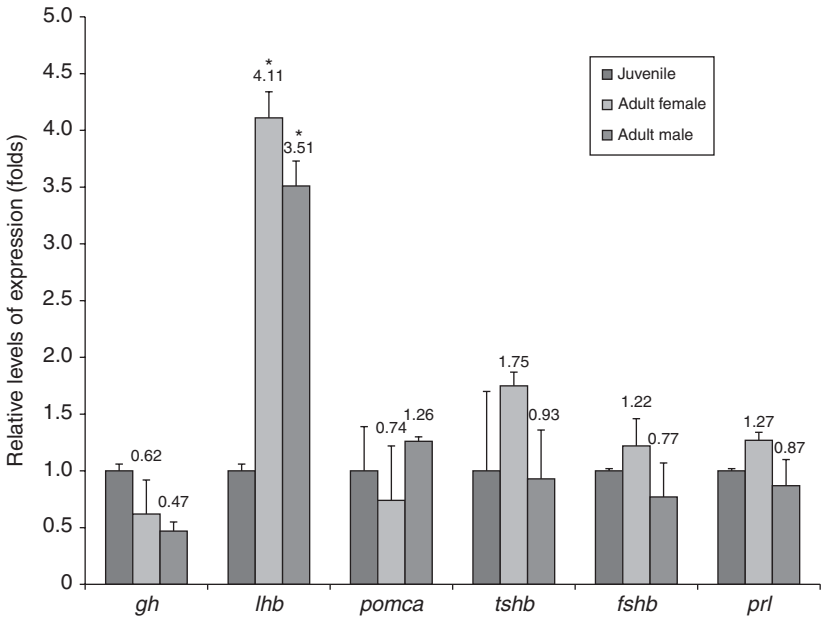

Figure 3

Results of the q-PCR analysis of the expression profiles of the major pituitary hormone transcripts in juvenile and adult male and female zebrafish. The transcriptional levels of growth hormone 1 (*gh*), proopiomelanocortin a (*pomc*), luteinizing hormone β-polypeptide (*lhb*), prolactin (*prl*), somatolactin-β (*smtlb*), thyroid-stimulating hormone β-subunit (*tshb*), and follicle-stimulating hormone, β-polypeptide (*fshb*) were measured. The mRNA levels of each of the hormone genes were normalized to β-actin1, β-actin2, and ribosomal protein L13a (*rpl13a*) as triple internal controls. For each gene at each time point, the values represent fold changes in expression compared with that in the pituitary gland of the juvenile fish, which were set at 1.0. The results are expressed as means ± S.D. ($n = 3$). *Significant difference between pituitary samples obtained from juvenile fish and those obtained from adult fish.

current Sanger sequencing- and microarray-based methods. Using the RNA-seq technique for the teleost pituitary transcriptome analysis, this study is the first to demonstrate that the sum of the eight major pituitary hormone mRNAs is equivalent to ~40% of the mRNA population in the pituitary gland at both juvenile and sexual maturity stages. The relative levels of the sums of the secreted signaling molecules, ribosomal proteins, and mitochondrial proteins remained consistent at 45 and 90 dpf (Table 2). This suggests that it may not be the drastic changes, but the relative ratios of the secreted signaling molecules in combination in the pituitary gland that are associated with the transition from the juvenile to the sexual maturity stage.

All the master endocrine glands and orthologs for nearly all the mammalian endocrine peptides have been identified in teleosts (Zohar *et al.* 2010, Kim *et al.* 2011, Lohr & Hammerschmidt 2011). However, the functional and anatomical correlations between many teleost and mammalian endocrine signals are still unclear. Our data indicated that the levels of *gnrhr2*, *lhb*, and gonadal steroid receptors, such as *ar*, *pgr*, *er1*, and *er2b*, were significantly higher in the pituitary gland of adult zebrafish (Table 4). Unlike in the case of mammals, it has been suggested that *lhb* expression in teleosts could also be directly regulated at the pituitary level by a series of neuroendocrine factors (Wong *et al.* 2006, Lohr & Hammerschmidt 2011). In addition to the observed expression patterns of the core hormone signaling molecules in the hypothalamic–pituitary–gonadal (HPG) axis, the expression levels of several other relevant neuroendocrine signaling molecules, including *grid1*, *gal*, and *drd1*, were also significantly elevated in the pituitary gland of adult zebrafish (Table 4). These are associated with Lhb production in mammals and/or fish (Flett *et al.* 1994, Wang *et al.* 2011, Garcia-Galiano *et al.* 2012, Fontaine *et al.* 2013). Our observations indicate that these signaling molecules might also be involved in the regulation of Lhb production in the zebrafish pituitary gland during the juvenile–sexual maturity transition.

In mammals, GH production is mainly under the dual control of GH-releasing hormone (GHRH) and somatostatin (SST) signals (Perez-Castro *et al.* 2012). Development from the juvenile to the sexual maturity stage marks the transition from a state of rapid body growth to a state of slow somatic growth (Gomez-Requeni *et al.* 2010).

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Unexpectedly, the transcriptional levels of *gh* in the pituitary gland remained the highest among the pituitary transcripts without a significant change at 45 and 90 dpf (Table 2 and Fig. 3). To understand the transcriptional regulation of *gh* in the pituitary gland, we analyzed the expression profiles of related signaling molecules. First, some significant changes in the expression levels of neuropeptide signaling molecules known to regulate *gh* expression, such as SST1, tandem duplicate 1 (*sst1.1*), neuropeptide Y (*npy*), and cholecystokinin (*cck*), were observed between the pituitaries at 45 and 90 dpf (Perez-Castro *et al*. 2012, Wong *et al*. 2006). However, these expression levels were relatively low (<4.0 RPKM, Supplementary Table 3). Second, the transcriptional levels of other neuropeptide signaling molecules, including Ssts and Sst receptors, Ghrh and Ghrh receptors, Npy receptors, thyrotropin-releasing hormone (Trh) and Trh receptors, corticotrophin-releasing hormone (Crh) and Crh receptors, and cholecystokinin (Cck) and Cck receptors, did not exhibit significant changes during the course of the transition from the juvenile to the sexual maturity stage (Supplementary Tables 1, 2 and 3). Lastly, virtually no reads of the ghrelin receptor were observed. Taken together, these results do not reveal a clear role of these factors in the regulation of *gh* transcription in the pituitary gland during this process. It has been demonstrated that changes in the gonadal steroid environment dramatically alter the patterns of GH secretion pulsatility from the pubertal stage to the adolescence stage in mammals. Therefore, the pulsatory patterns of Gh secretion, and not the transcriptional levels of *gh*, determine the levels of Gh in the plasma. Thus, gonadal steroids are the main factors that regulate the patterns of GH secretion (Sanchez-Cardenas *et al*. 2010). It has also been reported that sex steroids and some neurotransmitters, such as dopamine and glutamate, regulate Gh levels in fish sera (Wong *et al*. 2006, Canosa *et al*. 2007). In the present study, neither a significant stage- nor sex-specific difference in the transcriptional level of *gh* was observed (Figs. 2 and, 3, and Table 2). However, as shown in Table 4, the transcriptional levels of the gonadal steroid receptor, dopamine receptor, and glutamate receptor in the pituitaries changed significantly between the two stages examined, suggesting that the process of synthesis or secretion of Gh may be mainly regulated by gonadal steroids and some neurotransmitters in adult fish.

In addition to those in the HPG and somatotropic axes, we also found several significant changes in factors involved in the regulation of prohormone processing, immune responses, bone metabolism, and homeostatic control in the pituitary during the juvenile–sexual maturity transition. The expression levels of *Scg3* and *Scg5* were significantly higher at 90 than at 45 dpf (Table 3), indicating the active modulation of prohormone modification and endoproteolytic processing in the pituitary gland of adult zebrafish (Bartolomucci *et al*. 2011). Thymosins are important in the regulation of thymus development and in the induction of T-cell differentiation, both of which are vital for the immunological responses of organisms (Li *et al*. 2010). The transcriptional level of *tmsb15a* in the pituitary gland was dramatically decreased at 90 than at 45 dpf (Table 2). *Sparc*, *scpp7*, and *ogn* are important humoral bone anabolic factors involved in bone mineralization, formation, and remodeling (Choi *et al*. 2008, Delany & Hankenson 2009, Tanaka *et al*. 2012). The expression levels of these three transcripts decreased significantly from the juvenile to the sexual maturity stage (Table 2) and might be involved in the regulation of skeletal development and growth after sex maturation. Pituitary transcriptome analyses also provide an opportunity to understand the molecular events that contribute to the homeostatic control of pituitary cell growth. Several factors produced locally with an effect on pituitary cell proliferation have been identified. Among these factors, *igfs*, *il2*, and *pdgfa* promote pituitary cell proliferation (Table 4). By contrast, *tgfb* and *fst* inhibit the proliferation of specific cell populations in the pituitary gland (Wong *et al*. 2006, Saitoh *et al*. 2010, Perez-Castro *et al*. 2012). The transcriptional levels of these factors were significantly decreased in the pituitary at 90 dpf. In addition, a significantly lower level of the proliferating cell nuclear antigen (*pcna*) at the adult stage also indicated a decline in the proliferative index of the pituitary gland of adult zebrafish during the onset of adolescence (Table 4).

Profound gender differences exist in several aspects of the endocrine axes (Nishida *et al*. 2005, Sanchez-Cardenas *et al*. 2010). Thus, exploration of sexual dimorphic expression of the major hormones in the pituitary gland may help to understand the physiological regulation of growth metabolism in adult fish. Owing to the tiny size of the zebrafish pituitary, it is difficult to obtain a sufficient amount of total RNA from adult males and females separately for RNA-seq. Sexually dimorphic expression analyses on the pituitary tissue samples of adult fish were carried out using q-PCR. Among the six pituitary hormone genes, *lhb* was the only transcript with a significant change in expression pattern during the transition from the juvenile to the sexual maturity stage in zebrafish. These q-PCR analysis results were consistent with the RNA-seq

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analysis results. The q-PCR analysis data indicated that the six major pituitary hormone transcripts were differentially expressed to various degrees in adult male and female fish. However, there was no significant gender difference in the expression levels of each hormone transcript in the pituitary gland of adult fish (Fig. 3). Taken together, these results suggest that the functional regulation of the sexually dimorphic responses in the pituitary gland of adult fish might be mainly associated with the mechanisms of hormone modification and secretion through transcription-independent manners.

At the transcriptional level, characteristic changes included the transcriptional spurts of gonadotropin Lhb and gonadal steroid receptors during development. Our current studies focus only on gene expression profiling. It would be intriguing to carry out further integrative studies using other technical platforms, such as genetics and proteomic techniques. These would provide direct and comprehensive views of the dynamics and regulation of the biosynthesis and secretion of pituitary hormones. Further analyses of these and related pathways are expected to shed light on the intricacies of one of the most complex and intriguing developmental phenomena.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/JOE-13-0488.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
W H and X D performed the research; X C and J H analyzed and interpreted the data; and Z Y assisted and supervised the research team and wrote the paper.

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