Physiological studies of a unique endocrine gland in fish, named corpuscles of Stannius (CS), described a Ca\textsuperscript{2+}-regulatory function for this gland mediated by stanniocalcin-1, a hypocalcemic polypeptide hormone. However, to date, the endocrine functions of the glands have not been completely elucidated. We hypothesized that other unidentified active principles in the glands are involved in the regulation of plasma ion (Na\textsuperscript{+}, Ca\textsuperscript{2+}) and/or blood pressure. In this study, transcriptome sequencing of CS glands was performed using Japanese eels (*Anguilla japonica*) adapted to freshwater (FW) or seawater (SW) to reveal the presence and differential expression of genes encoding proteins related to the ion-osmoregulatory and pressor functions. We acquired a total of 14.1 Mb and 12.1 Mb quality-trimmed reads from the CS glands collected from FW and SW adapted eels, respectively. The *de novo* assembly resulted in 9254 annotated genes. Among them, 475 genes were differentially expressed with 357 up- and 118 down-regulated in the SW group. Gene ontology analysis further demonstrated the presence of natriuresis and pressor related genes. In summary, ours is the first study using high-throughput sequencing to identify gene targets that could explain the physiological importance of the CS glands.
with these reported physiological functions. In this study, a high-throughput transcriptome sequencing (RNA-seq) approach was adopted to investigate the transcriptome profiles of the CS glands from fish adapted to freshwater or seawater environments. The differential expression patterns of the CS glands were compared and the genes involved in Ca^{2+} metabolism, ion-osmoregulation, and blood pressure were identified. This study provides an important resource for future investigations on CS glands functions.

Methods

Maintenance of Japanese eels (Anguilla japonica). The methods were carried out in Hong Kong Baptist University in accordance with the approved guidelines. All experimental protocols were approved by the Hong Kong Baptist University, Hong Kong Special Administrative Region. Japanese eels (A. japonica) weighing between 500–600g, were reared in fiberglass tanks supplied with charcoal-filtering aerated tap-water (freshwater, FW) at 18–20 °C under a 12 h: 12 h L:D photoperiod for at least 2 weeks of acclimation before the experiments. The fish were then either maintained in FW (n = 5) or transferred to seawater (SW) (n = 5) for another two weeks. After this, the fish were anesthetized with 0.1% MS-222 (Sigma) for the collection of the CS glands.

RNA isolation, cDNA library Construction, and Illumina Deep Sequencing. Total RNA was isolated from the CS glands of fish using TRIzol reagent (Life Technologies, CA, USA). The RNA concentration was measured using Qubit® RNA Assay Kit in Qubit® 2.0 Fluorometer (Life Technologies, CA, USA). RNA samples (300 ng) with a RNA Integrity Number (RIN) greater than 8, as determined by the Agilent 2100 Bioanalyzer system (Agilent Technologies, CA, USA), were used for library construction. Four independent libraries were prepared for RNA sequencing. Briefly, the total RNA was purified and split into two equal aliquots. The libraries were then paired-end sequenced, using Illumina HiSeq 2500 with 2×150 bp paired-end reads. The base-calling was performed using Bcl2Fastq v1.8.4 and the resulting sequence data were submitted to the NCBI Sequence Read Archive (SRA) (http://www.ncbi.nlm.nih.gov/sra) under the accession number SRP049701.

Differential expression and GO enrichment analysis. In our analysis, differential gene expression and TMM-normalized FPKM gene expression were calculated separately. This is because RSEM does not support gapped alignment, and the alignment accuracy of Bowtie used by RSEM is known to be lower than that of other aligners, thereby hindering the use of the alignments produced by other aligners. Sequencing reads were mapped to the assembled transcripts using Novoalign (v3.00.05) with parameter -r ALL to report all multi-mapped reads (http://www.novocraft.com/). Alignment files were sorted using Samtools (http://samtools.sourceforge.net/) to generate a read-name sorted BAM file. Then, “Samtools view -F 0x4” was used to parse the mapped reads from the BAM file and the number of read-pairs mapping to each transcript in each sample were summarized to generate a count table. The reads were then processed using the RSEM V1.2.2 package to calculate the reads per kilobase per million mapped reads (RPKM) for each transcript. Dysregulated genes were subjected to KEGG pathway analysis using DAVID Tools to decipher the molecular interaction networks that might be deregulated.

Quantitative real-time PCR (qRT-PCR). To validate the sequencing data, an independent cohort of FW or SW (n = 3) adapted fish were sampled. The differentially expressed genes were selected for qRT-PCR analysis. Briefly, total cellular RNA (0.5μg) was reversed transcribed using the high capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA, USA). qRT-PCR reactions were conducted using the Power SYBR Green PCR master mix with the StepOne™ real-time PCR system (Life Technologies, CA, USA). Verified gene-specific primers (Table S1) of A. japonica were used. The occurrence of primer-dimers and secondary products was inspected using melting curve analysis. Our data indicated that the amplification was specific for each individual set of primers and control amplification was conducted either without reverse transcriptase or without RNA. gadph was used as a housekeeping gene and the relative expression ratio of target gene/gadph was calculated according to the method described by Pfaffl:

Expression ratio = \frac{E_{\text{treatment}}}{E_{\text{control}}} / \frac{E_{\text{treatment}}}{E_{\text{control}}},

where E_{\text{control}} was set to 1.

Availability of supporting data. The sequencing data from this study have been submitted to the NCBI Sequence Read Archive (SRA) (http://www.ncbi.nlm.nih.gov/sra) under the accession number SRP049701.

Results

Workflow of the study. In this study, a pair of CS glands from each fish was used to prepare one pooled RNA sample. Two biological replicates of FW and SW adapted fish were performed. Four cDNA libraries were constructed and subjected to Illumina transcriptome sequencing. The overall workflow of the study is shown in (Fig. 1).

Illumina RNA-Seq and de novo transcriptome Assembly. We obtained 7.72 Mb and 6.92 Mb quality-trimmed Illumina reads from the FW CS gland samples (FCS1 and FCS2, respectively) and, 6.61 Mb and 5.47 Mb quality-trimmed Illumina reads from the SW CS gland samples (SCS1 and SCS2, respectively). A total of 2.05 Gb and 1.73 Gb of clean bases were obtained from the FW and SW samples, respectively. The de novo transcriptome was formed by 78713 contigs with an average contig length of 791 bp (the shortest sequence was 201 bp and the longest one was 10424 bp) (Fig. 2).
**Gene annotation.** The assembled transcripts were subjected to 6-frame translations and the data of likely coding sequences were extracted. These likely coding sequences were randomized to provide a sequence composition corresponding to non-coding sequences. All the longest ORFs were scored according to the Markov Model (log likelihood ratio based on coding/noncoding) in each of the six possible reading frames. If the putative ORF proper coding frame scored positive and was the highest among the other presumed wrong reading frames, then that ORF was reported. If a high-scoring ORF was eclipsed by a longer ORF in a different reading frame, it would be excluded. Annotation analysis were implemented to compare the predicted ORF sequences against the UniProtKB/Swiss-Prot database using BLASTp search with a cut-off e-value of 1.0 × 10^-6. In this study, 9254 genes were matched to the UniProtKB/Swiss-Prot database (Table S2). Regarding the taxonomic distribution of the genes, according to the UniProtKB/TrEMBL database, 24.78% of the matched genes showed similarities with Lepisosteus oculatus, followed by Oncorhynchus mykiss (19.92%), Danio rerio (11.55%), Astyanax mexicanus (10.77%), Oreochromis niloticus (4.88%), Salmo salar (3.74%), Gasterosteus aculeatus (2.44%), Ictalurus punctatus (2.27%), Takifugu rubripes (2.10%), and others (15.95%) (Fig. 3). In fact, as of the date when the analysis was performed, only 1238 and 80 protein sequences of Anguilla species were deposited in the UniProtKB/TrEMBL and UniProtKB/Swiss-Prot databases, respectively. Therefore, it was not surprising to observe so few hits to Anguilla species, which was probably due to the under-representation of Anguilla species protein sequences in the UniProt database.

**Comparative analysis of A. japonica transcripts with various eel species.** We first compared our unified transcriptome assembly to the existing transcriptome resources of eel species. Eel endocrinology has been a subject of interest for a long time and, thus, numerous eel hormone sequences are available. To investigate what kind of hormones were expressed in the CS glands, we compared our assembled transcriptome to the existing eel protein sequences with hormone activity and found calcitonin, stanniocalcin, activin, adrenomedullin, insulin-like growth factor, natriuretic peptide, relaxin, urotensin, and ventricular natriuretic peptide in the CS gland transcriptome (Table S3). We then sought to study the expression of annotated eel genes in our CS gland-specific transcriptome. In order to accomplish this goal, we performed both nucleotide and protein level searches in our assembled transcriptome. The nucleotide search suggested that 39.2% of known transcripts discovered in various eel species are present in the CS gland of A. japonica (Table S4). We found that 27.8% of the proteins annotated in eel species by UniProt/TrEMBL non-reviewed database are present in our transcriptome assembly (Table S5).
The EeelBase database provides transcriptome resources of A. anguilla generated from 640,040 reads sequenced by both 454 and Sanger technologies\(^4,9\). We compared our A. japonica transcriptome assembly to that of the Eeelbase database using BLASTn search, with e-value <1E-5, identity ≥0.95, and number of aligned nucleotides ≥50%. It was found that 4085 A. anguilla transcripts annotated in the Eeelbase database are present in our A. japonica assembly. The Eeelbase database has also developed an Eeelbase specific microarray\(^39\) that targets a subset (~33%) of transcripts among their assembled A. anguilla transcriptome. The Eeelbase specific microarray targets A. anguilla transcripts that matched 2293 transcripts of our A. japonica assembly. Since the specificity of the microarray probe depends on the hybridization of the probe sequences to the cDNA to be probed, we required the alignment length threshold to be at least 90%, with an identity of at least 95%. We found that only 25% (~582) A. japonica transcripts could be probed uniquely by the Eeelbase specific microarray (Table S6).

Orthologous transcripts between A. japonica and A. anguilla were identified by comparing our assembled transcriptome to the predicted cDNA of the eel species. We decided against the use of only cDNAs from A. anguilla because of the low-coverage sequencing depth of the available A. anguilla transcriptome in the Eeelbase database. Based on reciprocal BLAST searches, 19382 putative homologs between the available A. anguilla and A. japonica transcriptome were identified (Table S7).

With the availability of the draft genomes of A. japonica and A. anguilla, we sought to determine the gene structure of our assembled A. japonica transcriptome by aligning the assembled transcripts to the draft genomes. Regarding the A. japonica genome, we found that the majority (89%; 69920/78713) of the transcripts could be aligned. In fact, 82% (64601/78713) of the transcripts were almost completely aligned (≥95% of the length of assembled transcripts). Regarding the A. anguilla genome, we found that the majority (78%; 61737/78713) of the transcripts could also be aligned. In fact, 70% (55078/78713) of the transcripts were almost completely aligned (≥95% of the length of assembled transcripts). Based on both A. japonica and A. anguilla transcript-to-genome alignments, the majority (~90%) of the transcripts expressed in the CS glands of A. japonica have 6 or less exons per transcript (Table S8).

**GO enrichment analysis and cluster classification.** All the genes were analyzed according to GO functional enrichment analysis (Table 1). The top five pathways involved in molecular functions included: GTPase regulator activity, nucleoside-triphosphatase regulator activity, nucleotide binding, small GTPase regulator activity, and ATP binding. The top five biological processes included: establishment of protein localization, protein localization, protein transport, intracellular transport, and regulation of small GTPase-mediated signal transduction. The top five cellular components identified in this analysis were: intracellular organelle lumen, organelle lumen, membrane-enclosed lumen, nuclear lumen, and nucleolus.

Furthermore, the genes of the CS gland transcriptome were classified into three clusters according to their functional annotation (Table 2). Cluster I included the genes involved in the regulation of calcium metabolism such as stanniocalcin-1 (STC-1), calcitonin, vitamin D(3) 25-hydroxylase, calcium-sensing receptor (CaSR), S100 calcium-binding protein A6 (S100A6), and stomatal interaction molecule 1 (STIM1). In cluster II, atrial natriuretic peptide (ANP)-converting enzyme and endothelin-converting enzyme 1 (EC-1) were listed. These enzymes are involved in the proteolytic cleavage of ANP and endothelin, respectively, to produce biologically active peptides that regulate blood pressure and natriuresis. In the cluster III are those transporters involved in ion-osmoregulation such as aquaporins, chloride intracellular channel protein 5, kidney-specific Na-K-Cl symporter, and voltage-gated potassium channel subunit Kv11.1.

### Table 1 | GO classification of genes from CS gland of Anguilla japonica.

| Term | gene | p-Value |
|------|------|---------|
| **Molecular function** | | |
| GTPase regulator activity (GO:0030695) | 168 | 6.20E-22 |
| nucleoside-triphosphatase regulator activity (GO:0060589) | 170 | 1.20E-21 |
| nucleotide binding (GO:0000166) | 616 | 1.50E-17 |
| small GTPase regulator activity (GO:0005083) | 118 | 5.10E-17 |
| ATP binding (GO:0005524) | 424 | 8.40E-15 |
| **Biological process** | | |
| establishment of protein localization (GO:0045184) | 258 | 3.40E-18 |
| protein localization (GO:0045184) | 287 | 4.10E-18 |
| protein transport (GO:0045184) | 254 | 1.60E-17 |
| intracellular transport (GO:0046907) | 217 | 2.50E-14 |
| regulation of small GTPase mediated signal transduction (GO:0051056) | 100 | 5.40E-12 |
| **Cell component** | | |
| intracellular organelle lumen (GO:0070013) | 503 | 9.60E-22 |
| organelle lumen (GO:0043233) | 506 | 3.30E-20 |
| membrane-enclosed lumen (GO:0031974) | 514 | 3.60E-20 |
| nuclear lumen (GO:0031981) | 418 | 1.60E-19 |
| Nucleolus (GO:0005730) | 216 | 3.10E-13 |
complex, basolateral plasma membrane, basal plasma membrane, endoplasmic reticulum, and basal part of the cell. Ten genes involved in the functional clusters were selected and validated by qRT-PCR analysis. Primers and amplicon sizes are listed in Table S7. The results of the qRT-PCR analysis agreed with the Illumina sequencing data (Table 4).

Discussion
The corpuscle of Stannius is a unique endocrine gland located on the ventral surface of kidneys of bony fishes. Although there is no comparable structure identified in humans, the mammalian ortholog of the CS-derived polypeptide hormone, stanniocalcin-1 (STC-1), was cloned and shown to be involved in many biological functions (i.e. ovarian physiology, inflammation, and carcinogenesis)

These results demonstrated the importance of the CS-derived factor in mammals, although the development of the glands disappeared during evolution. In past studies, STC-1 is the only polypeptide identified to be responsible for the role of CS glands in Ca2+ homeostasis. However, physiological experiments conducted in the past decades have also demonstrated ion-omoreregulatory and pressor functions of the glands while the identities of other CS-derived active principles, surprisingly, have not been elucidated to date. In this study, we sequenced CS glands isolated from FW or SW adapted fish, assembled the transcriptome, and identified differentially expressed genes.

Our primary goal was to identify genes that explain the reported physiological importance of the CS glands in the regulation of plasma ion (Na+, Ca2+) and/or blood pressure. Nevertheless, because of the availability of extensive transcriptome and genomic resources of a closely related species, A. anguilla, and the draft genome of A. japonica, we performed a comprehensive comparison between our assembled A. japonica transcriptome and these resources. We found that the following peptides with hormone activity were
expressed: stanniocalcin, calcitonin, activin, adrenomedullin, insulin-like growth factor, natriuretic peptide, relaxin, urotensin, and ventricular natriuretic peptide. Based on the experimental transcriptome assembly available in the EeelBase database, we estimated that more than 4085 A. anguilla transcripts are found in our A. japonica transcriptome. However, the EeelBase specific microarray may not be suitable for analyzing transcriptome-wide expression in A. japonica, primarily because only hundreds of A. japonica transcripts could be specifically hybridized to the array’s probe. Based on the transcriptome wide predicted cDNAs available in the ZF-Genomics database, we identified 19382 putative orthologous between the two widespread predicted cDNAs available in the ZF-Genomics database, we identified 19382 putative orthologous between

### Table 3 | GO classification of the differentially expressed genes from CS glands of Anguilla japonica.

| Term                                                                 | Count Total (up/down) | p-Value |
|----------------------------------------------------------------------|-----------------------|---------|
| **Molecular function**                                               |                       |         |
| Diacylglycerol binding (GO:0019992)                                   | 5(3/2)                | 2.21E-03|
| Calcium ion binding (GO:0005509)                                     | 14(11/3)              | 3.39E-02|
| Phospholipid:diacylglycerol acyltransferase activity (GO:0046027)   | 2(2/0)                | 3.98E-02|
| Cation binding (GO:0043169)                                          | 43(34/9)              | 5.49E-02|
| Ion binding (GO:0043167)                                             | 43(34/9)              | 6.79E-02|
| **Biological process**                                               |                       |         |
| cell adhesion (GO:0007155)                                           | 16(14/2)              | 4.79E-04|
| Biological adhesion (GO:0022610)                                     | 16(14/2)              | 4.86E-04|
| Cell part morphogenesis (GO:0032990)                                 | 9(8/1)                | 1.08E-03|
| Cell projection morphogenesis (GO:0048858)                           | 8(7/1)                | 3.65E-03|
| Homophilic cell adhesion (GO:0007156)                                | 6(5/1)                | 4.13E-03|
| **Cell component**                                                  |                       |         |
| Ubiquitin ligase complex (GO:0000151)                                | 7(6/1)                | 1.57E-04|
| Basolateral plasma membrane (GO:0016323)                             | 7(7/0)                | 1.00E-02|
| Basal plasma membrane (GO:0009925)                                   | 3(3/0)                | 2.27E-02|
| Endoplasmic reticulum (GO:0005783)                                   | 16(14/2)              | 2.50E-02|

*Quartile of the RNA-seq gene expression with p-value <0.05.

Asterisk indicates statistical significance of differential gene expression with p-value <0.05.

### Table 4 | Relative mRNA expression of 10 selected genes for comparison of the SW versus FW groups, in respect to RNA-Seq and real-time PCR.

| Gene                               | Transcript ID   | Real-time PCR (log2 fold change) | Illumina RNA-seq (log2 fold change) | Quartile of RNA-Seq gene expression |
|------------------------------------|-----------------|---------------------------------|------------------------------------|-------------------------------------|
| **Ca**⁺ metabolism                 |                 |                                 |                                    |                                     |
| Calcitonin-1                       | comp18601_c0    | 3.79 ± 0.19 *                   | 3.58                               | 75⁺                                 |
| Vitamin D(3) 25-hydroxylase        | comp7276_c0     | −5.73 ± 1.23 *                  | −3.37                              | 50⁺                                 |
| S100 calcium-binding protein A6    | comp22265_c0    | −1.58 ± 0.44 *                  | −3.66                              | 75⁺                                 |
| Tensin-2                           | comp12417_c0    | 1.72 ± 0.35 *                   | 1.99                               | 50⁺                                 |
| Stromal interaction molecule 1     | comp32767_c0    | 1.60 ± 1.04 *                   | 1.78                               | 25⁺                                 |
| **Ion-osmoregulation**             |                 |                                 |                                    |                                     |
| Chloride intracellular channel protein 5 | comp21102_c0 | −5.54 ± 1.72 *                  | −6.70                              | 75⁺                                 |
| Kidney-specific Na-K-Cl sympporter  | comp29031_c0    | 6.63 ± 0.40 *                   | 2.18                               | 75⁺                                 |
| Voltage-gated potassium channel subunit Kv11.1 | comp155490_c0 | 3.88 ± 0.47 *                   | 5.51                               | 50⁺                                 |
| Aquaporin-3                        | comp12261_c0    | −7.98 ± 3.06 *                  | −3.91                              | 50⁺                                 |
| NRip11                             | comp22263_c0    | 1.53 ± 0.64 *                   | 2.08                               | 50⁺                                 |

*Quartile of the gene expression of the group with higher expression. For example, Calcitonin-1 is induced by sea water treatment, and the quartile of average expression of SW was reported.

In addition to the general annotation, we addressed our particular research question using GO analysis to highlight the genes related to the three functional clusters: (1) Ca**⁺**-metabolism, (2) blood pressure, and (3) ion-osmoregulation. The differentially expressed genes under these three functional clusters were validated using real-time PCR analysis. In fish, STC-1 is known to be a hypocalcemic hormone involved in the regulation of Ca**⁺** homeostasis. However, the roles of other well-studied mammalian Ca**⁺**-regulating hormones (i.e. parathyroid hormone (PTH); calcitonin (CT); and 1, 25 dihydroxyvitaminD₃) in calcium metabolism in fish, are largely unknown. In mammals, CT is produced by parafollicular cells while in fish its presence was reported in ultimobranchial glands. In this study, we firstly identified the expression of CT in CS glands. A significantly higher CT expression level was...
detected in the CS glands of SW adapted fish. This observation implied that CT expression responded to high salinity and/or high ambient Ca\(^{2+}\) levels. In teleost fish, the regulatory role of CT in Ca\(^{2+}\) homeostasis is not conclusive. Some studies have shown an inhibitory action of CT on G\(_{\text{Gx}}\)AT in rainbow trouts\(^{44}\), suggesting a hypocalcemic function for CT. However, in another study, administration of CT caused hypercalcemia in brown trouts\(^{45}\). A recent study in zebrafish suggested that CT has a hypocalcemic function to inhibit ECAT expression\(^{46}\). In mammals, CT is one of the important hypocalcemic hormones, opposing the effects of PTH, exerting inhibitory action on osteoclast, and reducing intestinal and renal Ca\(^{2+}\) (re)absorption\(^{47,48}\). Nevertheless, the identification of CT expression in CS glands warrants further investigation of the role of CT in plasma Ca\(^{2+}\) homeostasis in fish. Besides CT, recently the PTH gene family was identified in the CS glands of a cartilaginous fish, the elephant shark\(^{49}\). One of the members, Pth1, was found to exert PTH-like activity in mammalian UMR106.01 cells and was believed to play a fundamental role in cartilaginous fish, before evolving to regulate bone development in teleosts. Surprisingly there was no Pth-like transcript detected. This observation implies that PTH-producing cells may have different developmental origin than CS glands\(^{50}\). In addition to the identification of hormonal factors, our data showed an increased expression level of the stromal interaction molecule 1 (STIM1) in the glands of SW adapted fish. STIM1, a Ca\(^{2+}\)-sensor in endoplasmic reticulum, mediates the activity of store-operated Ca\(^{2+}\) entry (SOCE) to regulate intracellular Ca\(^{2+}\) homeostasis. Upon Ca\(^{2+}\) depletion, STIM1 is translocated from the endoplasmic reticulum to the plasma membrane to activate Ca\(^{2+}\)-release-activated Ca\(^{2+}\) (CRAC) channel subunit\(^{51,52}\). A previous study in mammalian cells demonstrated that the function of the STC-1 paralog, STC-2, was to interact with STIM1 to negatively modulate SOCE\(^{53}\). Thereby, STIM1 may play a role in mediating the signal of extracellular Ca\(^{2+}\) to modulate STC-1 synthesis in CS glands.

In addition to the Ca\(^{2+}\)-regulatory function, early studies of CS gland physiology denoted the presence of pressor substances. In STX fishes, a decrease of dorsal aortic blood pressure was reported\(^{54}\). An injection of CS extracts increased blood pressure of fish\(^{55}\). However, the pressor substances in the glands that increased systemic blood pressure remain unknown. Through this transcriptomic analysis, we identified the expression of atrial natriuretic peptide (ANP) (ANP)-converting enzyme and endothelin-convertase enzyme 1 (ECE-1). Although no significant difference in their mRNA expression levels in CS glands was measured between the FW and SW adapted fish, the two enzymes are known to indirectly regulate blood pressure. The ANP-converting enzyme is an endopeptidase that cleavages atrial natriuretic peptide hormone into an active form to promote natriuresis and vasodilation\(^{56}\). ECE-1 is involved in proteolytic activation of endothelins, which have strong vasoconstrictive effects\(^{57}\). The identification of these important enzymes suggests the involvement of the glands in the regulation of blood pressure in fish and provides an explanation to the pressor effects of the gland extracts.

The functional cluster named "ion-osmoregulation" was formed by identifying the changes in the expression levels of the membrane transporters which were interpreted as the modulation of membrane sensors to integrate extracellular signals to regulate CS gland functions. In particular, the expression level of AQP-3 was significantly reduced in the CS glands of SW fish. The studies of the functions of water-specific, membrane-channel AQP proteins in mammals and fish suggested that AQPs have unique permeability characteristics, are widely distributed across tissues, and play important roles in the regulation of water homeostasis\(^{58,59}\). AQPs are functionally classified as osmotic-stress effectors. A long-term osmotic-stress in oysters induced a reduction of AQPs activities in response to osmotic challenges\(^{60}\). The reduced AQP-3 expression in the CS glands of SW fish might serve to similar functions.

In summary, our work represents the first report using next generation sequencing to identify gene targets that could explain the reported physiological importance of the CS glands. Three functional clusters were defined and differential gene expression was observed in the CS glands of fish adapted to FW and SW conditions. Taken together, our data support the notion that CS glands are important in the regulation of ion homeostasis and blood pressure. It warrants further investigation to decipher the underlying mechanisms that characterize the additional functions of this unique endocrine gland.

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Authors contributions
KPL participated in next generation sequencing experiments and drafted the manuscript, JWL and TFC carried out the transcriptome data analysis and drafted the manuscript, IG carried out real time PCR analysis and drafted the manuscript, WKFT participated in samples collection and samples preparation for transcriptome sequencing, CCKW participated in the design of the study and drafted the manuscript. JWL and IG contributed equally in this work. All authors read and approved the final manuscript.

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