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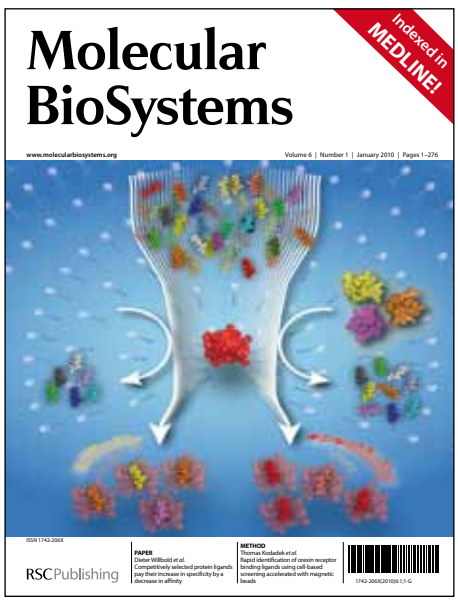

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Transcriptome and expression profiling analysis of *Leuciscus waleckii*: an exploration of the alkali-adapted mechanisms of a freshwater teleost

Yu-Mei Chang †[a], Ran Tang†[a], Xin-Jie Dou[a,b], Ran Tao[a, c], Xiao-Wen Sun[a] and Li-Qun Liang *[a]

a, Heilongjiang River Fisheries Research Institute, Chinese Academy of Fishery Sciences, Rd 43 Songfa, Daoli District, Harbin 150070, China

b, Fisheries and Life Science College, Shanghai Ocean University, Shanghai 201306, China

c, School of Marine Sciences, Ningbo University, Ningbo 315211, China

† These authors contributed equally

Abstract:

The strategies by which freshwater teleosts maintain acid-base homeostasis under alkaline stress are attractive and have been explored for a long time. In this study, a cyprinid fish that tolerates extremely alkaline environments (pH 9.6), *Leuciscus waleckii*, was used as a model to explore the molecular mechanisms of acid-base regulation. Using a lab-controlled alkaline challenge test and 454 sequencing, and the transcriptome of their gills and kidney were profiled and compared. mRNA profiling produced 1,826,022 reads, generated 30,606 contigs with an average length of 1,022 bp, of which 19,196 were annotated successfully. Comparative analysis of the expression profiles between alkaline and freshwater *L. waleckii* habitats revealed approximately 4,647 and 7,184 genes that were differentially expressed ($p<0.05$) in gills and kidney, respectively, of which 2,398 and 5,127 had more than twofold changes in expression. Gene ontology analysis and KEGG enrichment analysis were conducted. Comprehensive analysis found that genes involved in ion transportation, ammonia transportation, and arachidonic acid metabolism pathways changed dramatically and played important roles in acid-base homeostasis in fish under alkaline stress. These results support existing hypotheses about candidate genes involved in acid-base regulation under alkaline stress and prompt several new hypotheses. The large transcriptome dataset collected in this study is a useful resource for the exploration of homeostasis modulation in other fish species.

Introduction

Freshwater teleosts acclimate to intricate external conditions including alkaline water environments. The strategies used to maintain acid-base homeostasis under alkaline stress are attractive and have been explored for a long time. However, the studies of ionic regulation and acid-base homeostasis have been largely focused on euryhaline species, while research on fish in highly alkaline environments is limited[1-3]. Studies of the tilapia (*Alcolapia grahami*) in Lake Magadi (pH 10)[4] and the Lahontan cutthroat trout (*Oncorhynchus mykiss*) in Pyramid Lake (pH 9.4)[5] have mainly focused on physiology, biochemistry, but provided limited insight in the molecular mechanisms of alkaline adaptation[2, 6].

Terrestrial vertebrates alter their pulmonary ventilation to manipulate acid–base status[7], but the effect of this strategy is limited for fishes because of their low initial $PaCO_2$[6]. Acid-base regulation in fishes relies on direct transfer of acid-base equivalents[8]. Mitochondria-rich cells (MRCs) are widely distributed in the gill epithelium and maintain internal homeostasis via numerous transporters and channels that regulate the movement of ion and acid-base equivalents between plasma and the environment[8]. During periods of alkalosis, the base efflux (primary HCO_3^-) across the gill is generally increased, and the acid (H^+) efflux is reduced. Accordingly, the plasma HCO_3^- level decreases[6, 8, 9]. Physiological and pharmacological studies have proven that HCO_3^- excretion is commonly linked with Cl^- uptake and that H^+ excretion is linked with Na^+ uptake[10-12]. However, the molecular mechanisms are still incomplete, and the transporters involved in these bioprocesses are controversial. The anion exchanger (AE) is the primary mediator of apical Cl^-/HCO_3^- exchange in freshwater fish gills[9, 13-15], and SLC26[14] and SLC4[16] are the main contributors[13, 15, 17]. The carbonic anhydrase (CA) that catalyzes hydration reactions converting CO_2 to H^+ and HCO_3^-[18, 19] and the basolateral H^+-ATPase (HA) that extrudes H^+ to plasma are thought to create with HCO_3^--enriched microenvironments in MRCs. However, there is insufficient evidence for the co-expression of AE,

CA and HA[11]. Functionally, transporters involved in acid efflux also play important roles in maintaining internal homeostasis. For instance, the epithelial Na^+ channel (ENaC), HA, Na^+/H^+ exchanger (NHE) and basolateral Na^+-K^+-ATPase (NKA) are involved in Na^+ uptake and H^+ release[20-22]. In addition, the NH_3 channel Rhesus glycoproteins (Rh), which are responsible for NH_3/NH_4^+ excretion, drive the NHE by generating H^+ gradients[11, 23]. Furthermore, the Na^+/HCO_3^- co-transporter (NBC), chloride channels (ClC) and Na^+/Cl^--cotransporter (NCC) also play important roles in acid-base regulation in fish gills[11].

The kidney complements the gills in acid-base regulation. In freshwater teleosts, large volumes of urine are produced as a strategy for coping with the hypo-osmotic environment; therefore, renal transporters and channels responsible for ion reuptake and excretion are prerequisites[9]. The transporters NBC, NKA, HA and CA participate in renal HCO_3^- reabsorption and urinary acid excretion and are expressed in the kidneys of rainbow trout[24-26]. However, the molecular mechanisms by which these transporters regulate acid-base homeostasis in fish living in different environments are still unclear.

Leuciscus waleckii is a cold freshwater fish, and it is primarily distributed in water regions around the Amur River, a few areas in the Liao River, Yellow River, and inland lakes in Inner Mongolia in China[27]. One unique feature of this fish is its resistance to high alkalinity, which allows it to survive and adapt to Dali Lake, which is a saline-alkaline lake with a HCO_3^-/CO_3^{2-} concentration greater than 54 mM (pH 9.6) and the salinity greater than 0.6‰. In addition, *L. waleckii* in Dali Lake differ from their freshwater counterparts because they are characterized by breeding migration; they spawn in freshwater and grow in alkaline water[27]. There is a marked difference in the acute phase alkaline tolerance between the alkaline population from Dali Lake and the freshwater population from the Songhuajiang River, indicating the genetic heritability of alkaline tolerance[28].

Transcriptome profiling is a powerful strategy for identifying candidate genes and exploring their functions. Recently, EST sequencing and microarrays have been used to explore fish transcriptomes[29, 30], including those involved in acid-base regulation[31]. However, these traditional methods have limitation in depth, relative abundance of transcripts and genomic background[32]. In comparison, the rapidly developed high-throughput 454 sequencing technology provides deep-coverage RNA sequencing and a large number of ESTs[33, 34]. To explore the molecular mechanisms of alkaline adaptation, we sequenced the transcriptome profiles of the gills and kidneys of alkaline and freshwater *L. waleckii* on the 454 sequencing platform. The candidate genes and pathways associated with alkaline adaptation were analyzed, and the expression profiles of some key candidates were examined. Finally, the possible mechanisms linked to alkaline adaptation and future perspectives were discussed. This work generated a deep-coverage EST database and an overview of differentially expressed genes in *L. waleckii* experiencing alkaline stress.

Results

Transcriptome profiling of *L. waleckii*

L. waleckii in alkali (Dali) and freshwater (Songhuajiang) habitats were chosen for transcriptome sequencing. A batch of 1-year-old F1 progeny from a single mating of individuals from the alkali habitat was subject to alkali challenge in lab, while the F1 progeny of similar age from the freshwater habitat were used as a control group. Gills and kidney samples were taken from five individuals. A total of 12 samples, including 10 gills and 2 mixed kidneys, were sequenced independently, and the reads were combined for assembly in order to obtain the maximum number of ESTs. The sequencing results are summarized in Table 1. From a total of 1,826,022 initial reads, 142,406 nonsense reads were removed, leaving 1,683,616 high-quality reads with an average length of 295 bp. Of these, 1,509,049 reads were assembled into 30,606 contiguous sequences (contigs/isotigs) with an average length of 1,022 bp. The size distribution of these reads and contigs/isotigs is shown in Figure 1. The remaining 174,567 reads (singletons) were not assembled and were excluded from the subsequent data analysis.

From these 30,606 contigs, 30,590 ORFs were predicted using the GetORF program, and the predicted protein sequences were searched against the GenBank non-redundant protein (NR) database using the BLASTP program (E-value<0.001). As a result, 19,196 predicted proteins were annotated with their best matches. Most contigs (16,004) matched with proteins from zebrafish species; other hits came from other species including *Tetraodon nigroviridis* (401), *Salmo salar* (325), *Cyprinus carpio* (188), *Ctonopharyngodon idellus* (149), *Xenopus tropicalis* (126), and *Carassius auratus* (100). The remaining 11,394 predicted proteins failed to produce significant matches and therefore represented potential novel proteins.

To survey the different functional classes in the *L. waleckii* transcriptome, gene ontology (GO) analysis was conducted on all predicted proteins using GoPipe. Ultimately, 11,352 contigs were successfully grouped into generic GO terms (GO slims) and assigned to three categories. The contigs with GO terms corresponding to the

"biological process" grouped into 49 subcategories, "cellular component" into 33 subcategories and "molecular function" into 37 subcategories. The largest subcategory within the "biological process" group was "metabolic process", which contained 5,860 contigs and accounted for 13.2% of this group. In the "cellular component" group, the largest subcategory, "cell", contained 9,866 contigs and accounted for 20.9% of this group. The "binding" subcategory comprised 8,325 contigs (22.8%) and was the largest subcategory of the "molecular function" group. Subcategories that accounted for more than 1% of each group are summarized in Figure 2.

To analyze the active biological pathways in *L. waleckii*, contigs annotated with KO terms using the KEGG program revealed that 4,123 contigs mapped to 219 pathways. KEGG enrichment analysis using KOBAS 2.0 with the zebrafish control showed a total of 81 statistically enriched pathways grouped into 23 classes ($p<0.05$) (Additional Table 1); the distribution of KO terms in the enriched KEGG classes is shown in Figure 3. The KO terms were centralized in the classes related to "genetic information processing" and the "transport and catabolism", "immune system", "carbohydrate metabolism", and "lipid metabolism", classes, among others. In several pathways, the numbers of enriched KO terms were larger than the total numbers of KO terms detected in zebrafish, including the arachidonic acid metabolism pathway (23 with 7 additional KO terms), RNA transport pathway (106 with 50 additional KO terms) and mRNA surveillance pathway (52 with 29 additional KO terms). Pathways containing additional KO terms possibly have more specialized roles compared with zebrafish. The "proximal tubule bicarbonate reclamation", "collecting duct acid secretion" and "mineral absorption" pathways, which have functions related to ion uptake and acid-base equivalent transfers, were significantly enriched ($p<0.001$). The pathways involved in signal transduction, including "insulin signaling pathway", "phosphatidylinositol signaling system" and "notch signaling pathway", were also significant enriched in the *L. waleckii* transcriptome ($p<0.05$).

Comparative transcriptome analysis between freshwater and alkaline *L. waleckii*

To explore candidate genes whose expression changed remarkably between alkaline and freshwater habitats, the transcriptional changes of all contigs were evaluated in both organs. In gill samples, a total of 4,647 differentially expressed contigs ($p<0.05$) were found; of these, 892 contigs were up-regulated at least 2-fold and 1,506 contigs were down-regulated at least 2-fold. 535 up-regulated contigs and 1030 down-regulated contigs successfully annotated by the NR database were analyzed as the obviously variable contigs (Additional Table 2). In kidney samples, 7,184 contigs changed in transcriptional level ($p<0.05$), including 1,969 and 3,158 contigs that were up-regulated and down-regulated more than 2-fold, respectively. In total, 990 up-regulated and 2,240 down-regulated contigs were successfully annotated and considered differentially expressed (Additional Table 3).

GO classifications were assigned to the modulated contigs for functional annotation. Several alkaline-responsive contigs functionally related to ion transport were selected (Table 2), as they may be important for acid-base regulation. In gills, the expression of four Rhesus (Rh) glycoproteins in the "ammonium transport" were remarkably up-regulated, including Rhag (isotig11608), Rhbg (isotig05732), Rhcg1 (isotig02372) and Rhcg2 (isotig12999). TheRh family were considered to be involved in transporting ammonia and have been identified in several species of freshwater teleosts[35, 36]. Expressions of "sodium ion transport" groups were remarkably increased, including NCC (SLC12a3, isotig29555), the sodium/glucose co-transporter 5 like (SLC5a5l, isotig04892), the sodium-dependent ascorbic acid transporter (SLC23a2, isotig07010) and sodium/potassium ATPase (NKA alpha 1b polypeptide, isotig13741). The mRNA levels associated with base equivalent (HCO_3^-) transporters were also increased, and these contigs were classified with GO terms such as "anion transport" and "transmembrane transport", which include the known proteins AE1 (SLC4a1, isotig17702), Prestin (SLC26a5, isotig04059) and NBC (SLC4a4, isotig10404). In contrast, the expression levels of contigs functionally related to acid (H^+) transport were down-regulated, including NHE (SLC9a3, isotig16953) and genes in the "ATP synthesis coupled proton transport" GO group. Additionally, genes involved in Cl^-, K^+ and Ca^{2+} transport were differentially expressed at the same time. To confirm these transcriptional changes, the real-time qPCR were conducted in selected anion transporters (Figure 4A), sodium ion and H^+ transporters (Figure 4B), Rh transporters (Figure 4C). Relative expression changes derived from this transcriptome profile were also showed in Figure 4. Sperman's correlation test between data from different sources indicated that results from the real-time qPCR were consistent with the expression profiling analysis ($r=0.729$, $p=0.025$, $n=9$)[37].

In kidney tissue, most of sodium ion transport-related proteins were down-regulated, except for NKA beta 1b polypeptide (atp1b1b) and sodium/myoinositol cotransporter-like (slc5a3l) (Table 2, isotig10406 and isotig19395). NHE transcription (isotig16953) was increased, while the expression of other H^+ transporters was suppressed. Transcriptional levels of HCO_3^- transporters like anion exchanger 2 (SLC4a2) and NBC were up-regulated but Prestin (isotig17799) was down-regulated. As in gills, the genes involved in Cl^-, K^+ and Ca^{2+} transport were

differentially expressed.

Differentially expressed contigs were mapped to KEGG pathways to investigate the bioprocesses that were responsive to alkaline stress. A total of 660 contigs from gills were successfully annotated, with 465 KO terms and assigned to 218 pathways. In kidney samples, 1,531 modulated contigs were annotated with 1,082 KO terms and mapped to 262 pathways. The 33 and 47 statistically significant pathways ($p<0.05$) in gills and kidney, respectively, are listed in Additional Table 4. The 16 pathways enriched in both organs are shown in Table 3.

All KO terms in proteasome pathway that differentially expressed (23 in gills and 18 in kidney) were dramatically down-regulated. The proteasome is an essential component of the eukaryote ATP-dependent proteolytic pathway[38], which is responsible for the turnover of most cytosolic proteins. This mechanism regulates various cellular processes, such as DNA repair, chromatin remodeling, cell cycle regulation and modulation of signaling kinases[39, 40]. Most KO terms in three pathways belonging to the "translation" class were down-regulated which implied a global suppression of gene expression in both organs.

In addition, KO terms belonging to "Arachidonic acid metabolism" pathway were comprehensively regulated in two organs. This pathway mediates the conversion from Arachidonic acid (AA) into a series of metabolites which play important modulatory roles in living systems[41]. The differentially expressed genes of AA metabolism pathway in this study are shown in Figure 5. In gill tissue, the transcription of prostaglandin-endoperoxide synthase 1 (PES1), encoded by *ptgs1* (Additional Table 2, isotig20347), was up-regulated. PES1 converts AA to prostaglandin H2 (PGH2), which is a critical precursor for further synthesis of prostaglandins (PGs) including prostaglandin D2 (PGD2), E2 (PGE2), F2α (PGF2α), prostaglandin I2 (PGI2) and thromboxane A2 (TXA2)[42] . The stimulation of *ptgs1* indicating an enhancement of PGs production. However, mRNA level of *ptge3* (isotig14593) which encode prostaglandin E synthase 3 and mediate PGE2 production was decreased, implying the suppression of PGE2 synthesis. In the kidney expression profile of alkaline *L. waleckii*, PGD2 (encoded by *ptgds*, Additional Table 3, isotig16733) mRNA was up-regulated. In addition, for PG metabolism pathways, 5-HETE and 15(S)-HETE—the precursors of 5-Oxoeicosatetraenoic acid (5-OxoETE) and 15-Oxo-5, 8, 11-cis-13-trans-eicosatetraenoate (15-OxoETE), respectively, were stimulated, and genes related to the synthesis of other metabolites were down-regulated in gill and kidney tissue.

Discussion

The molecular mechanisms that regulate acid-base homeostasis have been explored in several fish model organisms. However, due to the differences between species and no direct confirmation of molecular similarities, pathways associated with fish acid-base regulation are incomplete, and the mechanisms are still ambiguous[2, 11]. The transcriptomic profiles of medaka (*Oryzias latipes*) response to alkalinity stress have been explored using gene microarray[31], candidate genes and biological pathways were identified. Although species and methods were totally different, the changing trends of some genes functionally related to ion regulation and metabolism were consistent with our study (like carbonic anhydrase XV, thioredoxin, S100 calcium binding protein A1, etc.). *L. waleckii* inhabits many ecotypes and has a high tolerance to alkalinity, it is an ideal model for exploring the mechanisms of alkaline resistance. Recently, EST sequencing of *L. waleckii* in nature environment of Dali Lake were conducted, the biological process involved in adaptive evolution was explored and genes under positive selection were identified [43]. Among which, several genes like interleukin-8 showed similar expression patterns to the profiles found in our work. In this study, the expression profiles of *L. waleckii* from alkali and freshwater habitats were compared, more concerns were focused on genes related to the acid-base regulation. The results showed that a large set of differentially expressed genes are mainly involved in biological and physiological procedures involving acid-base equivalent transport, ionic regulation, ammonia/urea excretion, and signal transduction pathways, among others.

Roles of ion transporters in the gills of alkali-adapted *L. waleckii*

Direct acid-base equivalent transport is the most important effector of acid-base homeostasis. The anion exchanger (AE) expressed in MRCs is involved in the direct transport of Cl^-/HCO_3^-, the transporter family SLC4 and SLC26 directly participate in this process[9, 44].

AE1 which belong to the SLC4 family was identified to locate on the apical membranes of MRCs and mediates HCO_3^- secretion[9, 15, 17]. In this study, alkaline *L. waleckii* was immersed in a sodium hydrogen carbonate ($NaHCO_3$) solution, which simulated an environment with high concentrations of Na^+ and HCO_3^-. The significant

up-regulation of AE1 strongly indicated its involvement in apical HCO_3^- secretion from epithelia to the surrounding environment, which was consistent with previous results in rainbow trout[17]. However, it is questionable whether AE1 is located on the apical surface of MRCs due to the use of non-specific antibodies[15]. Recently, it was shown that AE1 is located on the MRC basolateral membrane, which may facilitate HCO_3^- secretion from MRCs to plasma in zebrafish[16], as occurs in the kidneys of mammals[45]. Based on our results, this secretion would be impossible because the increased afflux of HCO_3^- in plasma is toxic to fish under alkaline stress. Even if AE1 were on the basolateral membrane, its function would have to be reversed (uptake the HCO_3^- from plasma) toward an inward HCO_3^- chemical gradient, as Romero (2004) postulated previously[46]. More evidence is needed to confirm either orientation.

Similar to the AE1, the location of NBC is closely related to its ion transport direction. Generally, NBC is expressed on the basolateral membrane of MRCs and facilitates Na^+/HCO_3^- co-secretion from MRCs to plasma under low-Na^+ stimulation[47]. In this study, the expression of NBC was induced, as measured by transcriptome analysis and confirmed by qPCR (Figure 4A). As noted above, stimulation of NBC expression enhances HCO_3^- transport into plasma, increasing plasma alkalinity to a potentially dangerous level for *L. waleckii* under alkaline challenge. Obviously, that scenario is not possible. The location of NBC was not only observed on the basolateral membrane of secreting related cells，in mammal it was also located on the apical membrane of epithelia in some organs like parotid and pancreas[48, 49]. Although the direct evidence in fish is absent, it is possible that NBC is expressed on the apical membrane of *L. waleckii* MRCs and drives Na^+/HCO_3^- co-secretion into water under alkaline stress.

The function of SLC26 family members in ion and acid-base regulation was also uncovered. Three members of the SLC26 family (SLC26a3, a4 and a6) were localized to MRCs in zebrafish, and their transcriptional levels were up-regulated during adaptation to a low-Cl^- environment[13, 14]. In our study, four members of the SLC26 family (SLC26a1, a5, a6 and a11) were expressed in *L. waleckii*, but only SLC26a5 (Prestin) transcription was up-regulated obviously (more than 3.5-fold) under alkaline induction, as confirmed by real-time qPCR (Figure 4A). Prestin has been previously thought to be a motor protein that generates electromotility rather than a transporter[50]. Recently, its functions in mediating electrogenic divalent/chloride exchange in chicken and zebrafish[51, 52] and in Cl^- uptake and HCO_3^- secretion in *Drosophila* and *Anopheles*[53] have been discovered. According to these observations, we postulated that Prestin is a new candidate member of the SLC26 family and that it mediates base secretion when fish are confronted with alkaline stress.

Transport of H^+ and other metal ions across gill epithelium is also involved in acid-base homeostasis[9]. NHE and HA are putative transporters involved in apical H^+ secretion, as they are expressed on MRCs [21]. The roles of these two candidates in Na^+ uptake and H^+ secretion are still debated [54]. They may be expressed differentially depending on the environmental situation. Generally, HA is induced to mediate H^+ secretion in low pH, while the expression of NHE was stimulated to mediate Na^+ uptake in a low-Na^+ environment[55]. Our results showed that the expression of NHE3 (SLC9a3, certified by subsequent experiments, Figure 4B) and HA (V-type H^+-ATPase) were both significantly down-regulated, indicating that there are low levels of Na^+ uptake and H^+ secretion when *L. waleckii* is under alkaline stress. NCC mediates Na^+ and Cl^- uptake in the MRCs of rainbow trout and zebrafish in freshwater[11, 56, 57]. In *L. waleckii*, transcriptional level of isotig29555 was up-regulated dramatically which was annotated as the NCC, implying that Na^+/Cl^- uptake was stimulated under alkaline stress. However, NCC has four isoforms that expressed in fish, all of which are belong to the SLC12 family[58]. In zebra fish, another isoform (SLC12a10.2) but not SLC12a3 was identified as gill-specific expression, therefore, it still needs further exploration to certify the certain isoform of NCC in gills of *L. waleckii.* NKA is an important component in the whole mechanism of fish ionic and acid-base regulation, which mediates Na^+/K^+ transport and drives other transporters[9, 21, 59]. Likewise, the up-regulation of NKA alpha1b (Table 2, isotig13731) was observed in *L. waleckii* under alkaline stress, further indicating the important role of NKA in acid-base regulation.

Roles of ammonia transporters in the gills of alkali-adapted *L. waleckii*

Ammonia excretion by freshwater teleosts is another key adaptation to alkaline habitats. Ammonia (NH_3 or NH_4^+) in fish is a form of nitrogenous waste that is produced from excess amino acid degradation and AMP deamination [60]. The accumulation of ammonia is highly toxic to fish [61]. While generally not a problem in normal and low pH environments, ammonia excretion becomes a major problem for fish exposed to high pH or alkalinity due to the reduced gradient of NH_3 diffusion between plasma and the environment [43]. In some freshwater teleosts inhabiting alkaline environments, distinctive strategies of ammonia excretion are keys to their survival[60, 62]. Because nearly all cell membranes have very low permeability to NH_3 or NH_4^+, fish expresses channel proteins in certain cell types to enhance the movement of ammonia across plasma membranes[60]. The branchial epithelium is the major

site of ammonia excretion[8, 60], where Aquaporin (AQP) and Rh proteins are involved in it[63, 64]. In *L. waleckii* gills, the expression of three aquaporins was down-regulated or unchanged (Additional Table 2, AQP3, isotig13725, down-regulated 2.1-fold), but four Rh homologs were up-regulated, indicating that Rh proteins rather than AQPs were important for ammonia excretion and acid-base regulation in alkali-adapted *L. waleckii*. In addition, V-ATPase and/or NHE form a metabolon with Rh proteins to promote apical ammonia excretion (acid trapping) in freshwater teleosts[22, 65]. Surprisingly, these two transporters were down-regulated (V-ATPase B1 subunit, isotig 12165, down-regulated 3.3-fold; NHE3, isotig16953, down-regulated 2.8-fold) in *L. waleckii* gills. This result suggests that some unknown transporter mediates H^+ transport, or, alternatively, that ammonia excretion in *L. waleckii* is independent of H^+ secretion. Therefore, interesting areas of future research include the unknown H^+ transporter proteins and the mechanism by which Rh proteins transfer ammonia excretion in a highly alkaline environment without acid trapping.

Roles of ion transporters in the kidneys of alkali-adapted *L. waleckii*

There is much less knowledge of acid-base retention mechanisms in kidneys than of those in gills. The key component of this mechanism is the adjustment of urinary acid excretion by manipulating the re-absorption of filtered HCO_3^- so that it diffuses into the urea [9, 66]. Membrane-associated carbonic anhydrase catalyzes the conversion of H^+ and HCO_3^- into CO_2 in the proximal tubule, then CO_2 diffuses into tube cells and hydrates into HCO_3^- and H^+, and finally HCO_3^- is re-absorbed into plasma via special transporters [9, 66]. V-ATPase and NHE3 are involved in apical H^+ excretion[9, 67], as their mRNA levels are increased during acidification[68, 69]. In this study, the mRNA expression levels of several isoforms of V-ATPase were decreased, indicating the suppression of renal H^+ secretion in highly alkaline water to a level that matched the decreasing HCO_3^- re-absorption. In contrast, the expression of the Na^+/H^+ transporter-like protein was up-regulated, and a search for homologs confirmed that this isotig was part of an NHE3 sequence. Although this result needs to be confirmed, the function of NHE3 here is likely to be different from other species, where it acts as an H^+ excluder[66, 69]. NBC is another transporter on the basolateral membrane of renal tube cells. It mediates HCO_3^- transport into plasma, and its mRNA levels are increased during hypercapnic acidosis[68]. Likewise, NBC expression in *L. waleckii* kidneys was induced (Table 2, isotig10404) by the reversed condition (high-HCO_3^-), a result that indicated its possible involvement in alkaline adaptation and hinted at its different roles in renal acid-base regulation. Anion exchanger 2 (AE2, SLC4a2) is expressed on the apical membrane of pronephric duct epithelial cells and mediates Cl^-/HCO_3^- exchange[70]. The expression of AE2 in *L. waleckii* kidneys was stimulated during alkalosis (Table 2, isotig20200), which was inconsistent with previous observations in a mammal[71]. Considering the decreased expression of Prestin, AE2 may be a better candidate for active HCO_3^- transport in the *L. waleckii* kidney. The expression of a number of ion transporters were down-regulated, including Rhcg (isotig06579), SLC5a2 (isotig09584), SLC5a9 (isotig10124), $Na^+/K^+/2Cl^-$ co-transporter (isotig13043), SLC12a1-like (isotig19779), SLC12a2 (isotig24638). The overall decreased mRNA levels reflect a wide suppression of ion transport in kidney tissue during alkaline stress.

KEGG pathways involved in the alkaline stress response

KEGG enrichment analysis of differentially expressed contigs/isotigs indicated the potential bio-processes mediating the alkaline response. In this study, KO terms in proteasome pathway and translation related pathways were down-regulated comprehensively in both organs. This change strongly indicated a global inhibition of both protein degradation and synthesis and implied a stagnancy of metabolic processes for *L. waleckii* responsive to alkaline stress. The abundant stimulated genes observed in this work confirmed that such suppression should not be a nonspecific process, it may focus on the metabolic processes which uninvolved in the acid-base homeostasis, energy economization is the possible purpose.

According to our analysis, AA metabolism pathway from both organs was affected in *L. waleckii* during alkaline stress, which suggested a possible “universal role” in acid-base regulation. In fish, the AA metabolism pathway mainly operates in the gills, opercular membrane and kidney. Some of its products control various physiological functions through adjusting hormone release and/or altering target organ sensitivity[72, 73]. AA and/or its metabolites are involved in the osmoregulatory process in several teleosts[72, 74, 75], but its role in acid-base homeostasis in fish is unclear. Previous research considered PGs as the important local signal agents synthesized in teleost gills. They modulate ion transport across the epithelium directly or by altering the size and distribution of transport cells[76]. Furthermore, their concentration is affected by osmotic stress[77]. Base on the gene expression changes shown in Figure 5, we hypothesize that the increased expression of PES1 stimulated the production of PGs, which were subsequently involved in local signal transduction in acid-base regulatory pathways during alkalosis. Interestingly, PGE2 production were suppressed when synthesis of other prostaglandins (including PGD2, PGI2 and TXA2) were unaffected. Presumably, other molecules generated by PGH2 but not PGE2 metabolism were involved in the

AA cascade during alkaline stress. PGD2 is widely researched in the field of inflammation and disease in mammals; it mediates decreases in membrane permeability in human cells[78]. Therefore, stimulation of PGD2 synthesis in kidney may regulate HCO_3^- re-absorption by adjusting the permeability of epithelial cell membranes. Besides PGs, other regulated metabolites such as HETE and DHET are likely signals for the acid-base regulation mechanism. The AA metabolism pathway may be involved in endocrine signal transduction during acid-base homeostasis regulation.

Materials and methods

Fish and alkaline challenge experiment

The wild alkali-adapted *L. waleckii* were caught from Dali Lake in Inner Mongolia, and their freshwater counterparts were caught from the Songhuanjiang River, which is a branch of the Amur River. The alkaline or freshwater *L. waleckii* offspring were obtained in the lab from a single mating. A total of 30 alkaline 1-year-old F1 (mean weight, 100 g) were subjected to alkaline challenge in the lab, and the same number of freshwater F1 were kept in freshwater until sampling. During the acclimation, feeding was stopped for two groups of fish, which were maintained in flow-through water conditions at a relatively constant water temperature of 20°C. For alkaline F1, a sodium hydrogen carbonate solution was added and titrated to affect an approximate 10 mM increase in alkalinity every 3 days. Over the course of 15 days, the alkalinity was gradually increased to a final concentration of 50 mM (pH 9.0), and alkaline F1 were maintained in these conditions for 7 d. All surviving individuals in each group were anesthetized, and gill and kidney samples were stored in RNAlater (Ambion, USA). Five individuals from each group, comprising 12 samples including 10 gills and 2 mixed kidneys, were selected for 454 sequencing. For qPCR, both organs from six other individuals in each group were analyzed.

RNA isolation, cDNA synthesis and 454 sequencing

Tissue samples were disrupted by a Retsch mixer mill MM400 (Retsch, German), and the total RNA was purified with TRIzol® Reagent (Invitrogen, USA) according to the manufacturer's protocol. The integrity and quantity of the extracted RNA were determined using an Agilent 2100 (Agilent, USA). cDNA was synthesized with GsuI-oligo dT primer, Superscript II reverse transcriptase (Invitrogen USA), Dynal beads M280 (Invitrogen USA), DNA ligase and Ex Taq polymerase (TAKARA, Japan), as Ng (2005) described[79]. cDNA was fragmented to 300-800 bp by sonication and purified with Ampure beads (Agencourt, USA). Single-strand template DNA (sst DNA) libraries were constructed with a GS DNA Library preparation kit (Roche, USA). Finally, cDNA was amplified with a GS emPCR kit (Roche, USA) and sequenced on a Roche 454 GS FLX system[80].

Gene Assembly, annotation and expressional analysis

Raw reads generated from 454 sequencing were filtered of primers, adaptors, poly A/poly T tails, low quality sequences and very short (<100bp) sequences (nonsense reads) by using SeqClean (http://sourceforge.net/projects/seqclean/) and Lucy (http://lucy.sourceforge.net/) program. The filtered high-quality reads were assembled by Newbler V2.3 (http://www.454.com/products/analysis-software/) using sequence type "cDNA" with the "extend low depth overlaps" parameter. As a result, 30,606 contigs were obtained. To annotate these contigs, we predicted the putative ORFs of all contigs using an in-house developed program based on "GetORF" from EMBOSS[81], and then searched the predicted amino acid sequence against the NCBI NR database using BLASTP. The best matches, with E-values smaller than 0.001, were used for the annotation. Gene ontology (GO) was predicted with GoPipe using the Swiss-Port and TrEMBL database[82], and functional annotation with the KOG database was also performed. KEGG ontology (KO) was annotated on the KOBAS 2.0 platform (http://kobas.cbi.pku.edu.cn/). To analyze the expression profile, we counted the number of reads in each contig as log2-transformed RPKM[83] and then used the method MARS (MA-plot-based method with Random Sampling model) of DEGseq package [84]. The contigs with p-values smaller than 0.05 and more than 2-fold changes in expression were considered significant.

Real-time qPCR analysis

cDNA from each sample was synthesized using the High-cDNA Reverse Transcription Kit (Applied Biosystems, USA), according to the manufacturer's protocol. The gene specific primers are listed in Additional Table 5. The SYBR green qPCR Master Mix (Applied Biosystems, USA) was used to quantify the expression of each target gene on a quantitative PCR machine (ABI 7500, Applied Biosystems, USA). A two-step real-time RT-PCR program was conducted as follows: enzyme activation step at 95°C for 5 min and 45 cycles of 95°C for 15 s, 59°C for 15 s and 60°C for 25 s. The 18s ribosome RNA was used as an internal control [85]. Data was collected and analyzed using the $2(-\Delta\Delta Ct)$ method [86].

Conclusion

The mechanisms of fish resistance to environmental stresses are interesting biological questions. We selected an extremely alkali-tolerant population of a freshwater teleost (*L. waleckii*) to study acid-base regulation by high-throughput deep sequencing. This study included the first large-scale RNA sequencing of freshwater teleosts inhabiting a highly alkaline environment. The transcriptome and gene expression profiles revealed that alkaline *L. waleckii* was highly sensitive to external stimuli. To avoid alkalosis, its internal systems responded quickly and actively by dramatically changing the expression of multiple genes involved in ion and ammonia transport, and signal transduction. The large transcriptome dataset collected in this study is a very useful resource for the exploration of acid-base homeostasis in other fish species.

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Figure legends

Figure 1. The size distribution of reads (a) and contigs/isotigs (b) generated by 454 RNA sequencing.

Figure 2. Gene ontology (GO) ID representations for the *L. waleckii* transcriptome. Three comparisons are shown: (a) Biological process; (b) Cellular component; (c) Molecular function.

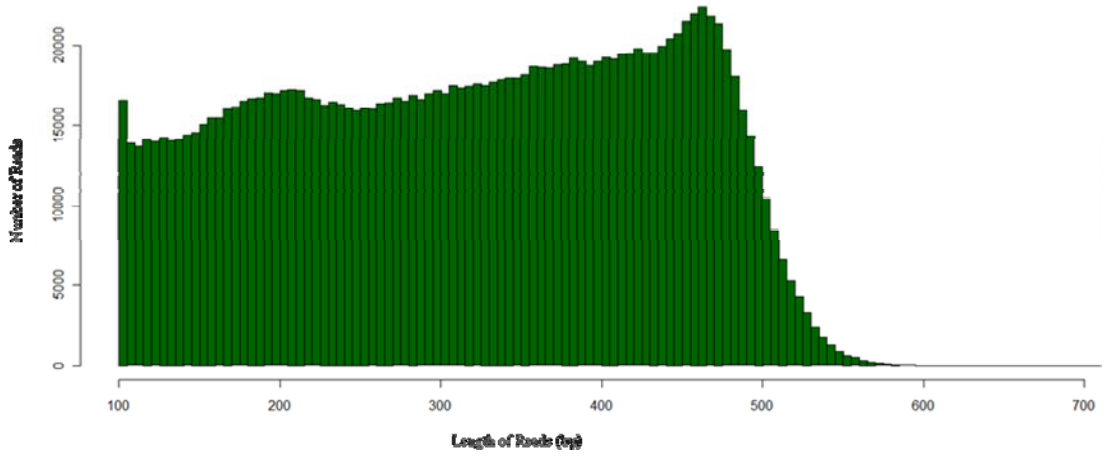
Figure 3. Distribution of KO terms in enriched KEGG classes. The abscissa represents the total number of KO terms in each class.

Figure 4. The expression analysis of selected genes by quantitative real-time PCR. Total RNA was extracted from the gills of Dalinor population fish adapted to alkaline water and Heilongjiang population fish reared in fresh water. Quantitative real-time PCR was used to validate the differential expression of HCO_3^- transporters (A), Na^+ transporters (B) and ammonium transporters (C). Expression changes relative to the control 18S gene are shown. For each gene, the gray bar indicates the gene expression ratio of the Dalinor population adapted to alkaline water validated by real-time PCR. The values are expressed as means ± SD from three different experimental replicates. The white bar indicates expression ratio derived from the transcriptome profile. The black bar indicates the expression ratio of the Heilongjiang population reared in fresh water, where the value is defined as 1. Statistical significance of the relative expression ratio is indicated (*, Student's *t*-test, $p<0.05$)

Figure 5. Genes involved in the arachidonic acid metabolism pathway. The pathway map depicts the KEGG arachidonic acid metabolism reference pathway (map00590). The rectangles indicate enzymes encoding genes, and circles indicate metabolites. The color in the upper half of the rectangle reflects the gene expression changes in gills, and the lower half reflects the change in kidney. Red indicates significantly increased expression (>2-fold change, $p<0.05$); blue indicates significantly decreased expression (<-2-fold change, $p<0.05$); white, unchanged; gray, undetected in this trancriptome.

Figure 1

(a)



(b)

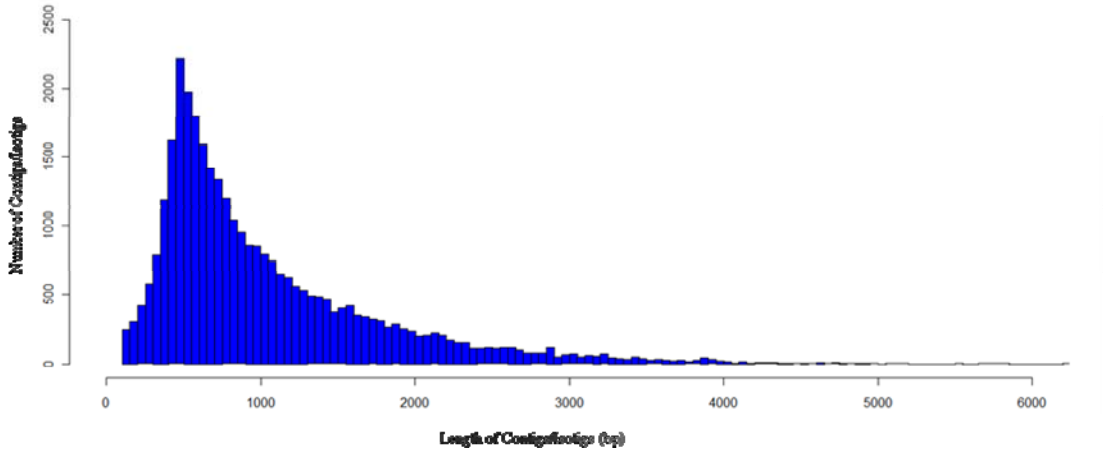
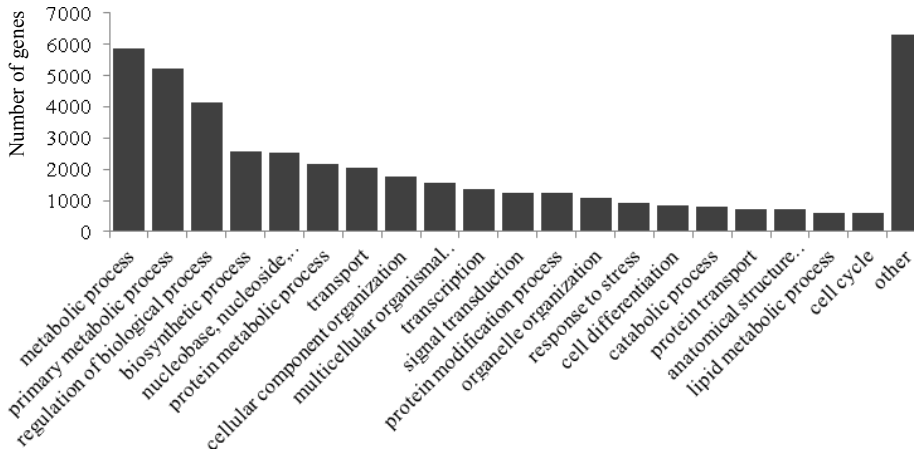
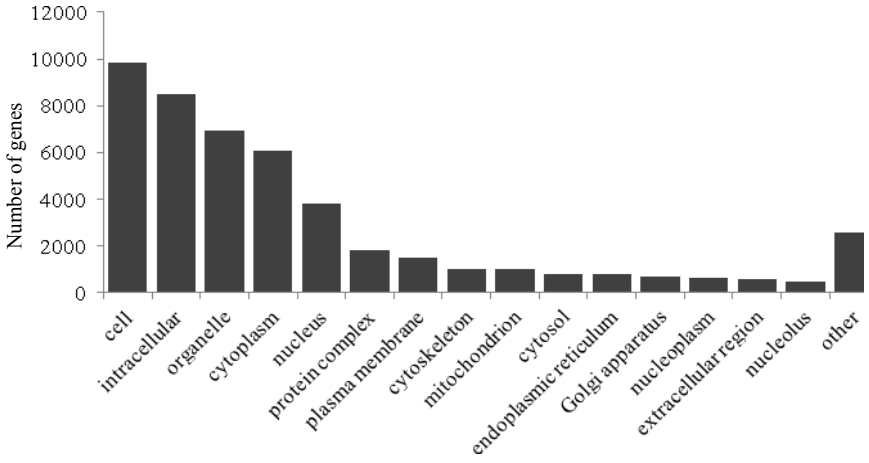


Figure 2

(a)



(b)



(c)

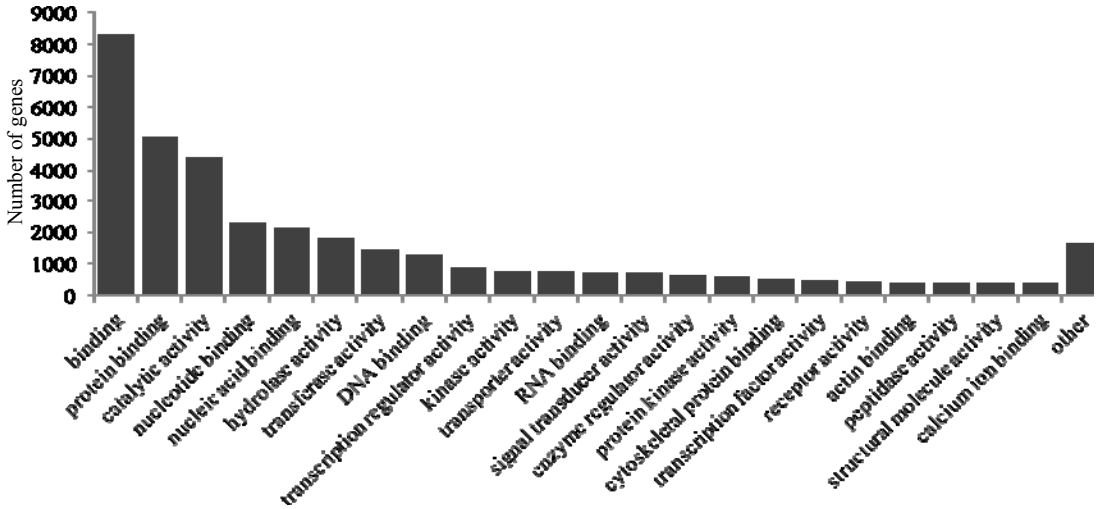


Figure 3

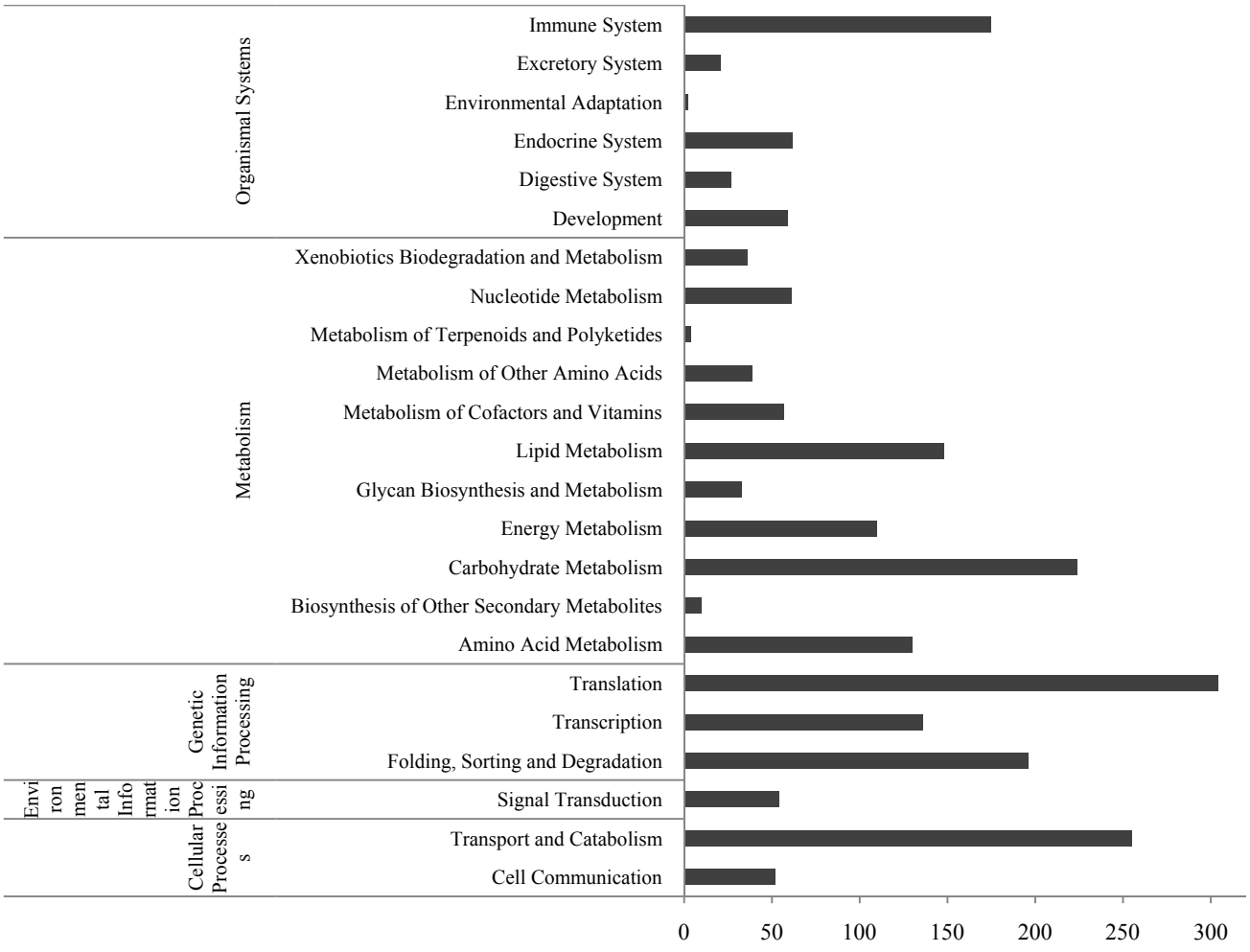


Figure 4

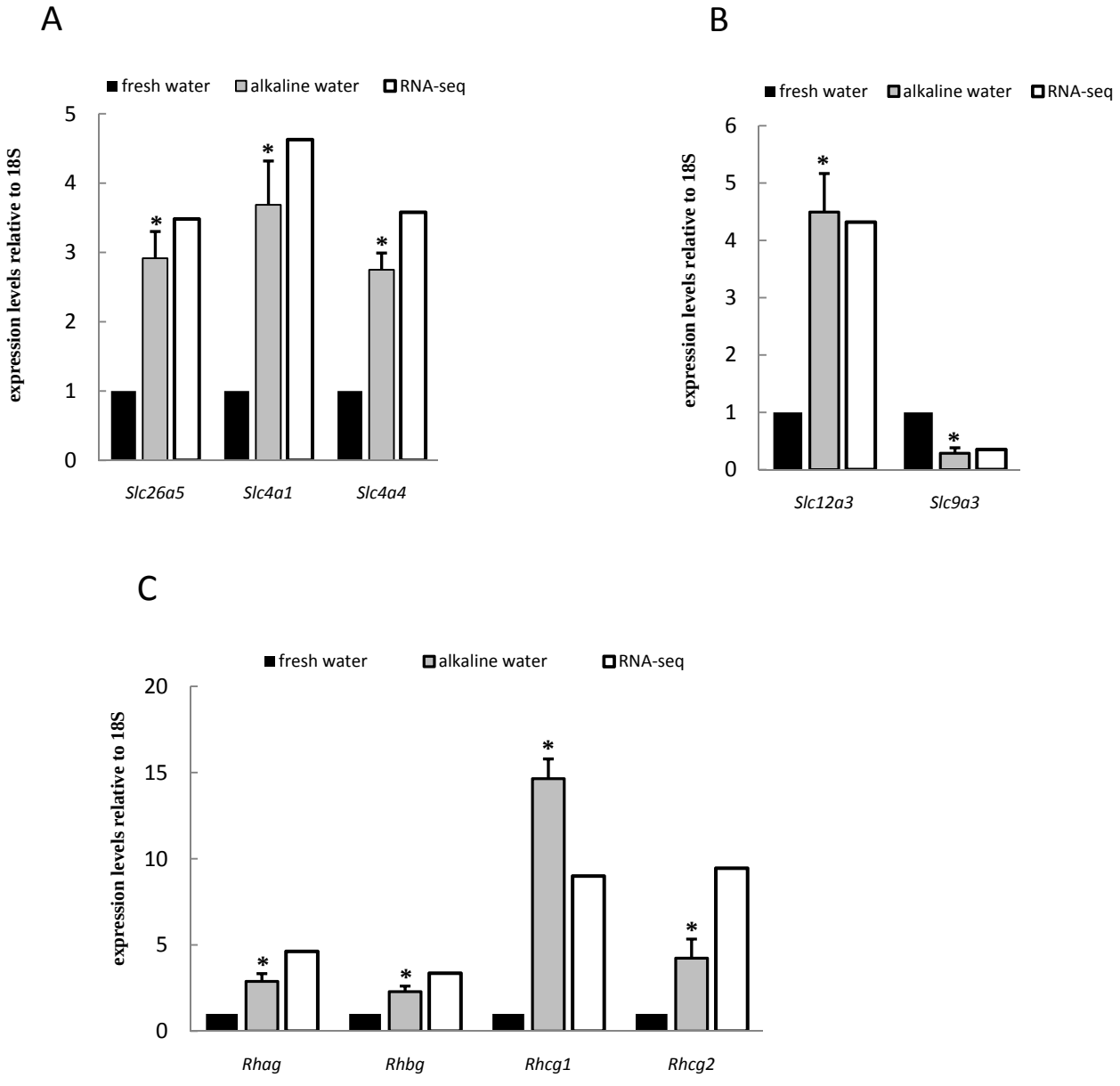


Figure 5

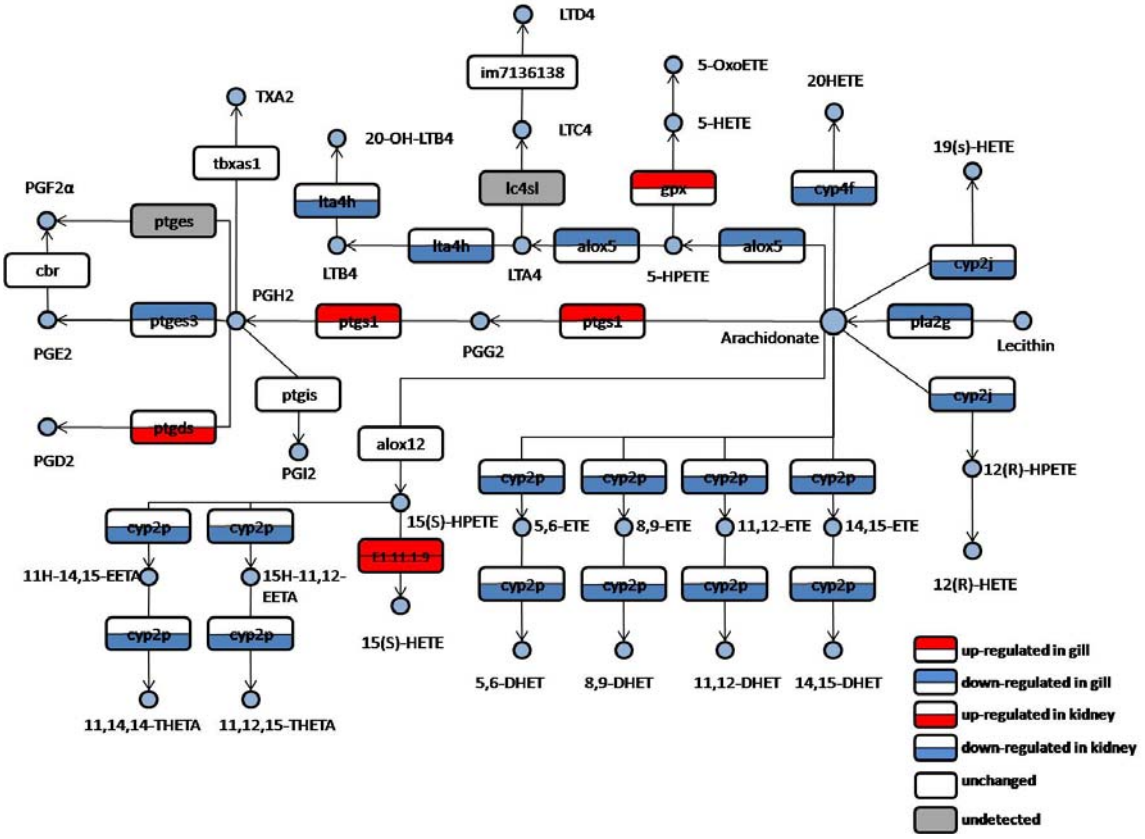


Table 1. Summary statistics of 454 sequencing

	AW*	FW**
Total Reads (n)	955,655	870,367
Q20 percentage	92.5%	92.3%
High Quality Reads (n)	881,925	801,691
Average Reads Length (bp)	295	
Assembled Contigs (n)	30,606	
Average Contigs Length (bp)	1,022	
Singlets (n)	92,663	81,904

*****AW indicates samples isolated from the Dali population adapted to alkaline water.

******FW indicates samples isolated from the songhuajiang population reared in freshwater.

Table 2. Representative contigs that are functionally related to ion transport and are differentially expressed in gills and kidney

Gill			Kidney		
Sequence ID	log FC*	NR annotation	Sequence ID	log FC*	NR annotation
GO:0015696 ammonium transport					
isotig11608	2.21	Rhesus blood group-associated glycoprotein	isotig06570	-1.93	Rh type C glycoprotein1
isotig02372	3.17	Rhesus blood group, C glycoprotein a			
isotig12999	3.24	Rh type C glycoprotein2a			
isotig05732	1.75	ammonium transporter Rh type B			
GO:0006814 sodium ion transport					
isotig16953	-1.51	novel protein similar to vertebrate solute carrier family 9 (sodium/hydrogen exchanger) member 3	isotig04206	-1.21	sodium-coupled monocarboxylate transporter 2
isotig29555	2.11	novel protein similar to vertebrate solute carrier family 12 (sodium/chloride transporters), member 3 (SLC12A3)	isotig09584	-3.16	novel protein similar to vertebrate solute carrier family 5 (sodium/glucose cotransporter), member 2 (SLC5A2) (zgc:85792)
isotig13741	1.22	ATPase, Na+/K+ transporting, alpha 1b polypeptide	isotig10124	-3.16	Slc5a9 protein
isotig04892	1.53	PREDICTED: sodium/glucose cotransporter 5-like	isotig10405	-2.69	Solute carrier family 38, member 7
isotig07010	1.21	PREDICTED: solute carrier family 23 member 2	isotig10406	1.08	ATPase, Na+/K+ transporting, beta 1b polypeptide
			isotig13043	-2.03	putative Na/K/2Cl cotransporter
			isotig15355	-2.4	PREDICTED: solute carrier family 22 member 5-like
			isotig16953	1.3	novel protein similar to vertebrate solute carrier family 9 (sodium/hydrogen exchanger)
			isotig19395	3.34	PREDICTED: sodium/myoinositol cotransporter-like
			isotig19779	-2.4	PREDICTED: solute carrier family 12 member 1-like
			isotig24638	-3.69	solute carrier family 12 member 2 isoform 1
			isotig28776	-2.57	sodium/myo-inositol cotransporter 2
			isotig29321	-4.16	Slc5a9 protein
GO:0006821 chloride transport					
isotig07537	-1.3	chloride intracellular channel protein 2	isotig11506	-1.22	PREDICTED: chloride channel protein 2
			isotig31242	-4.16	PREDICTED: calcium-activated chloride channel regulator 4
GO:0006816 calcium ion transport					
isotig15689	4.28	plasma membrane calcium-transporting ATPase 1	isotig13195	-2.1	stromal interaction molecule 1
isotig15688	-1.03	Sorcin	isotig13263	-1.02	coronin-1A
isotig13263	-1.06	coronin-1A	isotig15343	-2.31	Purinergic receptor P2X, ligand-gated ion channel, 4a
			isotig15815	5.47	stanniocalcin
			isotig16105	1.91	PREDICTED: two pore calcium channel protein 1
			isotig16476	-2.16	PREDICTED: inositol 1,4,5-trisphosphate receptor type 2-like
			isotig19534	-2.57	'Inositol 1,4,5-trisphosphate receptor type 1'
			isotig20385	-2.69	PREDICTED: inositol 1,4,5-trisphosphate receptor type 1
			isotig25776	3.82	ATPase, Ca++ transporting, plasma membrane 1b
GO:0006820 anion transport					

isotig17702	2.21	Solute carrier family 4, anion exchanger, member 1	isotig20200	1.71	solute carrier family 4, anion exchanger, member 2a
isotig10404	1.48	sodium bicarbonate cotransporter	isotig10404	1.34	sodium bicarbonate cotransporter
GO:0006813 potassium ion transport					
isotig12848	2.39	potassium inwardly-rectifying channel, subfamily J, member 1	isotig13046	-2.57	BTB/POZ domain-containing protein KCTD5
GO:0015986 ATP synthesis coupled proton transport					
isotig18985	-1.58	ATP synthase subunit g, mitochondrial	isotig04699	-1.49	Vacuolar ATP synthase subunit e 1
isotig11078	-1.72	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit b, isoform 1	isotig05023	-1.21	PREDICTED: v-type proton ATPase catalytic subunit A
isotig05534	-1.25	Zgc:101757 protein	isotig12292	-1.12	V-type proton ATPase subunit d 1
isotig04979	-1.84	T-cell immune regulator 1	isotig13095	-1.57	novel protein (zgc:63516)
isotig12165	-1.73	vacuolar-type H+ transporting ATPase B1 subunit	isotig13230	-4.07	ATPase, H+ transporting, lysosomal, V1 subunit H
			isotig16501	-1.1	ATP synthase subunit gamma, mitochondrial
			isotig20839	-1.69	ATP synthase, H+ transporting, mitochondrial F1 complex, delta subunit
			isotig21001	-1.03	ATP synthase subunit d, mitochondrial
GO:0055085 transmembrane transport					
isotig04059	1.8	prestin	isotig17799	-1.23	novel protein similar to solute carrier family 26, member 5 (slc26a5)
GO:0015840 urea transport					
isotig13411	2.39	urea transporter 2			
GO:0006879 cellular iron ion homeostasis					
isotig25876	-3.23	vacuolar fusion protein MON1 homolog A	isotig01773	-1.19	ferritin heavy chain
isotig02119	-3.9	PREDICTED: nidogen-2	isotig12193	4.39	ceruloplasmin

* log fold change

Grouped according to GO (slim) terms. Contigs that were annotated by more than one item in this table were assigned to one term only.

Table 3. Pathways statistically enriched with differentially expressed genes in both gills and kidney

Class	Pathway	gills		kidney	
		KO No.*	P-value	KO No.*	P-value
Amino Acid Metabolism	Phenylalanine, tyrosine and tryptophan biosynthesis	1/1	0.00322	1/1	0.03739
Biosynthesis of Other Secondary Metabolites	Tropane, piperidine and pyridine alkaloid biosynthesis	1/1	0.00322	2/0	0.03739
	Caffeine metabolism	0/1	0.02564	1/1	0.01123
Carbohydrate Metabolism	Glycolysis / Gluconeogenesis	1/6	0.00360	4/6	0.0327
Digestive System	Pancreatic secretion	5/3	0.01807	3/11	0.0415
	Mineral absorption	1/2	0.02188	2/6	0.00016
	Carbohydrate digestion and absorption	2/1	0.03798	4/4	0.00091
Excretory System	Proximal tubule bicarbonate reclamation	1/1	0.02267	2/1	0.04897
Folding, Sorting and Degradation	Proteasome	0/23	4.90E-15	0/18	0.00056
Immune System	Complement and coagulation cascades	1/7	0.01131	20/3	2.81E-07
	Fc gamma R-mediated phagocytosis	0/9	0.00993	2/16	0.003
Lipid Metabolism	Arachidonic acid metabolism	2/4	0.00037	2/6	0.00335
Signal Transduction	MAPK signaling pathway – yeast	1/1	0.02267	0/3	0.04897
Translation	Ribosome biogenesis in eukaryotes	0/7	0.00062	2/15	4.97E-09
	RNA transport	1/15	9.70E-05	4/22	0.00051
	Ribosome	0/17	0.00028	0/27	0.00425

*Number of KO terms in each pathways and organs is shown as the form like “number 1/ number 2”, “number 1” on left of symbol “/” indicates number of expressional up-regulated KO terms, while “number 2” on the right indicates the down-regulated KO terms number.

Additional Table 1. Statistically enriched KEGG pathways in *L. waleckii*

Additional Table 2. Differentially expressed contigs in the gills of *L. waleckii*

Additional Table 3. Differentially expressed contigs in the kidney of *L. waleckii*