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Letter to the editor Open Access

Genetic basis of embryo and juvenile physiological responses to salinity changes in freshwater pipefish (*Hippichthys heptagonus*)

DEAR EDITOR,

Syngnathidae species commonly inhabit ocean environments. However, some have adapted to live exclusively in freshwater over long-term adaptive evolution but continue to retain physiological adaptations to saltwater environments. The genetic basis underlying the adaptive strategies and molecular regulation of freshwater syngnathids to freshwater and saltwater remains unclear. Here, we investigated the molecular characteristics and core gene expression in freshwater belly pipefish (*Hippichthys heptagonus*) embryos and juveniles through salinity stress experiments and transcriptome analysis. Results showed that embryonic exposure to salinity at a concentration of 30‰ down-regulated cell cycle-associated genes vital to embryonic development. Retinol metabolism, neuroactive receptor interaction, and peroxisome proliferator-activated receptor signaling pathways were significantly enriched in up-regulated genes in the embryos. Notably, there was no significant change in the expression of ion transport and energy metabolism genes. Conversely, juvenile exposure to 30‰ salinity up-regulated ion transport-related genes and significantly enriched immune-related signaling pathways, including lysosome, phagosome, autophagy, and mitophagy signaling pathways. Carbohydrate metabolism genes were also up-regulated, whereas oxidative phosphorylation genes were significantly down-regulated. These results suggest that brood pouch protection during the embryonic stage and salinity adaptation plasticity in juveniles may be strategic adaptations in freshwater pipefish.

Environmental salinity regulates physiological function, growth performance, and survival rate in many marine organisms, and energy allocation is a balance between osmoregulation and growth expansion (Bœuf & Payan 2001; Hora et al., 2016). Salinity variation can change the expression patterns of genes involved in immune and osmotic responses (Goehlich et al., 2021). Male pipefish have a specialized brood pouch, which is important for gas and waste transportation, osmoregulation, and immune protection for embryos during male pregnancy, as observed in male seahorses (Li et al., 2020; Zhang et al., 2020).

Belly pipefish (*H. heptagonus*), which mainly inhabit shallow freshwater rivers and/or brackish estuaries, incubate their eggs in a brood pouch attached in the belly prior to gestation (Figure 1A). We previously explored the tissue structure and evolutionary traits of the brood pouch in freshwater Manado pipefish (Zhang et al., 2020), providing some insight into genetic regulation during salinity-induced stress. Therefore, in the present study, we focused on the molecular mechanism of *H. heptagonus* embryos and juveniles under different levels of salinity exposure. Transcriptomic analysis was employed to reveal the gene regulation and interaction processes of pipefish under different treatments.

A total of 33 pregnant pipefish were cultured under three different salinity treatments (0, 15, and 30‰, respectively) for six days after acclimation. Embryos were collected from the brood pouches of three pregnant pipefish per treatment. Subsequently, one-day old juveniles born from pregnant pipefish in each treatment were cultured for another six days in 0, 15, and 30 ‰ tanks, then sampled for transcriptomic analysis. Paired-end reads were generated via sequencing using the Illumina HiSeq 2500 platform. Raw data were filtered using in-house Perl scripts, and clean data were obtained by removing adapter sequences and low-quality reads. Sequence quality control was based on Q30 quality scoring and GC content (Supplementary Table S1). In total, 102.50 Gb of clean data were obtained and paired-end sequencing reads were mapped and aligned to the belly pipefish genome (data not published). Annotated and new genes were identified for further analysis. Principal component analysis based on the first two components indicated that the embryos in the low- and high-salinity groups were distinct from samples in the three juvenile groups (Supplementary Figure S1). We identified 1 442 (599 up-regulated and 843 down-regulated), 2 489 (1 198 up-regulated and 1 291 down-regulated), and 2 011 (904 up-regulated and 1 107 down-regulated) differentially expressed genes (DEGs) in embryos under 30 ‰ treatment (0‰ vs. 30‰) and in salinity-stimulated juveniles (0‰ vs. 15‰ and 0‰ vs. 30‰), respectively (Supplementary Figure S2).

Embryos had fewer DEGs overall. The top 10 KEGG signaling pathways enriched in DEGs in embryos and juveniles between the 0 ‰ and 30 ‰ groups were noted (Figure 1A). In embryos, the purine metabolism (ko00230),

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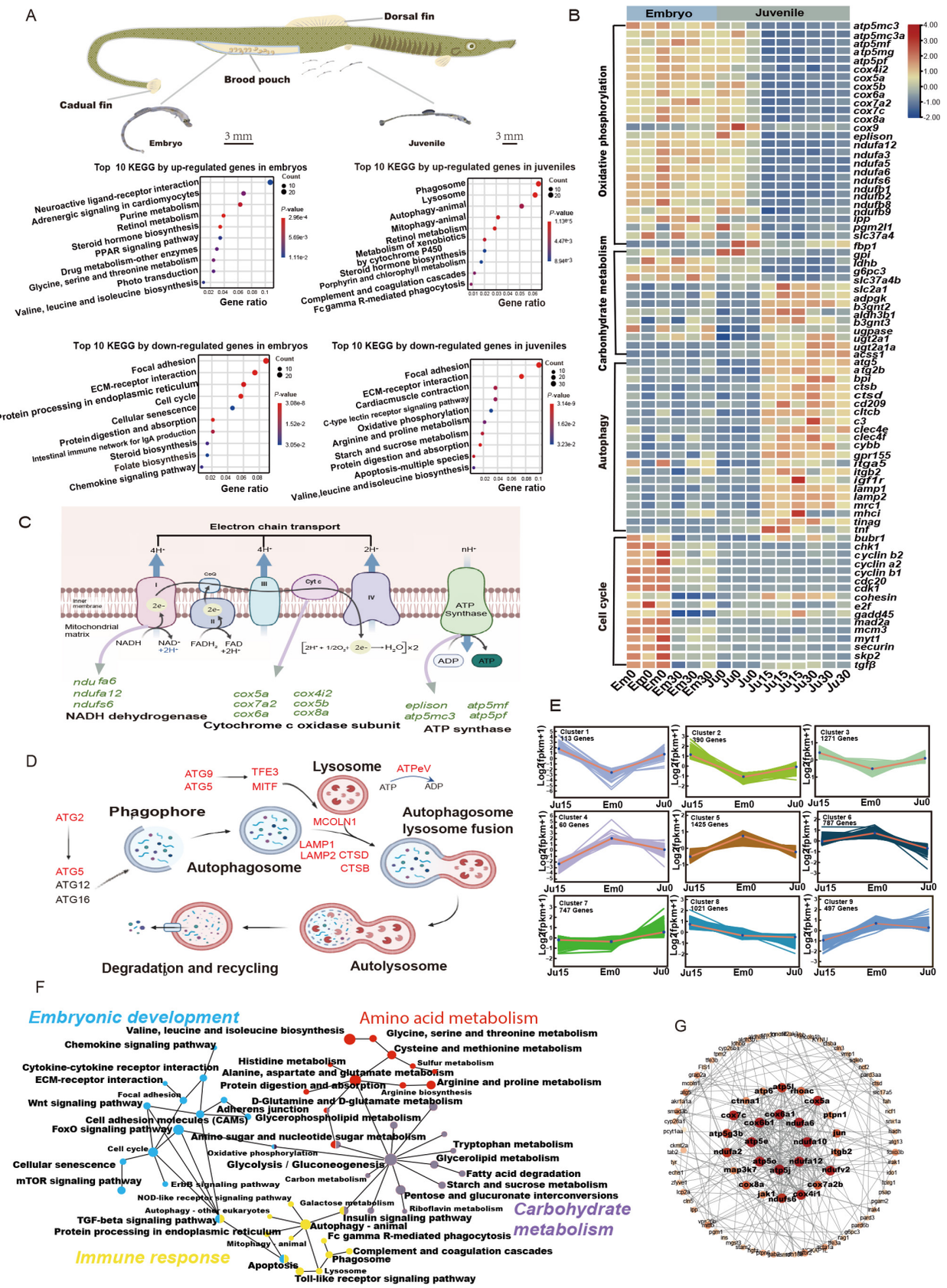

Figure 1 Transcriptomic analysis results

A: Profiles of pregnant belly pipefish, embryos, and juveniles and signaling pathways enriched in DEGs in embryos and juveniles (both from 0‰ vs. 30‰). B: Heatmap of genes involved in oxidative phosphorylation, carbohydrate metabolism, autophagy, and cell cycle. C, D: Schematic of signaling pathways related to oxidative phosphorylation and autophagy, respectively. E: Expression pattern analysis and gene clusters. F: Relationship between signaling pathways and classification of different signaling pathway clusters. G: Protein-protein interaction (PPI) network analysis, where node size and color gradient represent level of connectivity.

retinol metabolism (ko00830), glycine/serine/threonine metabolism (ko00260), and peroxisome proliferator-activated receptor (PPAR) signaling pathways (ko03320) related to metabolism were enriched in up-regulated genes. In our study, pathways related to embryonic development, including focal adhesion (ko04510), ECM-receptor interaction (ko04512), and cell cycle (ko04110), were significantly enriched in down-regulated genes (Figure 1A). Moreover, high salinity also impacted signaling pathways related to amino acid metabolism and protein-related pathways, such as protein processing in the endoplasmic reticulum (ko04141) and protein digestion and absorption (ko04974). In juveniles, five of the top 10 signaling pathways enriched in up-regulated genes were related to immune responses (Figure 1A), including phagosome (ko04145l), lysosome (ko04142), autophagy (ko04142), complement and coagulation cascades (ko04610), and phagocytosis (ko04666). The down-regulated genes were involved in oxidative phosphorylation (ko00190). Juvenile exposure to 15‰ showed similar expression patterns as the 30 ‰ treatment group (Figure 1B; Supplementary Figure S3).

Although embryos are protected by the brood pouch, changes in salinity can still affect embryonic development. High salinity may inhibit cell proliferation and differentiation in embryos. Collagen is involved in the focal adhesion and ECM receptor interaction signaling pathways. In our study, collagen and integrin family genes, which regulate cell migration and differentiation, were down-regulated (Supplementary Figure S4A, B). Eighteen genes were significantly enriched in the cell cycle signaling pathway, which is important for embryonic development (Figure 1B). The down-regulation of these genes can limit cell cycle processes. Notably, *cdk1* can inhibit the expression of *drf1*, a replication initiation factor, and low *drf1* expression is essential for embryonic development (Collart et al., 2017). Thus, the down-regulation of *cdk1* in the pipefish embryos may result in high *drf1* expression, which, in turn, inhibits the cell cycle signaling pathway. *Cyclin b*, *cyclin a1*, and *cyclin a2* can bind to *cdk* kinases, such as *cdk1*. Previous studies have reported that *cyclin a2* improves embryo quality in gilt-head sea bream (Georgiou et al., 2022), and low *cyclin a2* expression can affect embryonic development. Furthermore, neuroactive ligand-receptor interactions play important roles in neurosystem development in animals (Wei et al., 2020). Proteins encoded by the up-regulated *grm6*, *grin3b*, and *grin2b* genes are receptors for these compounds (Supplementary Figure 1C). The up-regulation of *rdh1*, *rdh3*, and UDP-glucuronosyltransferase family genes, which are involved in retinol metabolism, may be important for eye development during the early developmental stages (Supplementary Figure S4D) (Gao et al., 2020). Several genes involved in fatty acid metabolism, including *acsl1*, *cyp27a1*, and *cyp7a1*, were up-regulated (Supplementary Figure S4E). Cyp27a1 and cyp7a1 are vital for transferring cholesterol into bile acids, and acsl1 can degrade bile acids into free fatty acids, which provide energy for embryonic development (Gao et al., 2020). Therefore, our findings suggest that varying salinity levels may influence embryonic development in belly pipefish.

Osmoregulation is a basic physiological process for energy expansion in marine organisms. The electron transport chain is a metabolic system in the mitochondria, in which energy is generated under different enzymes and proteins encoded by *ndufa12*, *ndufs6*, and *ndufa6* contribute to NADH generation, an important element of oxidative phosphorylation. Cytochrome c oxidase is a subunit protein encoded by *COX* genes and *atp5mf*, *atp5mc3*, *atp5pf*, and epsilon form the components of adenosine triphosphate (ATP) synthase and are important genes in the oxidative phosphorylation signaling pathway. In our study, genes related to oxidative phosphorylation were down-regulated in juveniles maintained under hypersaline conditions (Figure 1B), consistent with the effects of salinity on other species, such as euryhalines, in which salinity changes can affect energy supply (Guo et al., 2018). Moreover, carbohydrate metabolism genes were differentially expressed in juveniles exposed to high-salinity stress, including the up-regulation of genes related to glycolysis (Figure 1B). *Slc2a1* is a member of the gene family associated with facilitating glucose transport. ADPGK catalyzes D-glucose phosphorylation, yielding adenosine diphosphate (ADP) to generate ATP for energy support. The *b3gnt2*, *b3gnt3*, *aldh3b1*, *ugpase*, *ugt2a1*, *ugt2a1a*, and *acss1* genes contribute to glycolysis, which is involved in gluconeogenesis (Figure 1B). Thus, high salinity may promote glycolysis, with the energy supply from this biological process compensating for the deficiency caused by salinity-induced changes in oxidative phosphorylation.

We predicted that a high-salinity environment may trigger an immune response. High expression of autophagy-related genes (*ATG*) and cathepsin D (*CTSD*) is related to the autophagy signaling pathway. Previous studies have confirmed that the innate immune response is critical in the early stage of vertebrate development (Vaz et al., 2022). Autophagy can facilitate protein turnover, as it contributes to the removal and regeneration of aging cells and organelles. In our study, *ATG* family genes (*atg5*, *atg2b*, and *atg9*) were highly expressed in the autophagy signaling pathway, and *CTSD* facilitated autophagosome and lysosome fusion (Figure 1B, D). Furthermore, genes involved in immune signaling pathways, including *itga5*, *itgb2*, *igf1r*, *mrc1*, *mhc1*, *tinag*, and *tnfa*, were up-regulated in juveniles, whereas genes related to immune responses were not significantly expressed in the embryos. This suggests that the brood pouch may protect embryos from environmental stress.

Genes associated with osmoregulation were differentially expressed in juveniles exposed to high salinity (Supplementary Figure S5). Additionally, genes encoding glucocorticoid receptor 1 (gr1), glucocorticoid receptor 2 (gr2), insulin-growth like factors 1, 3, and 5, and insulin growth factor were up-regulated. The DEGs related to ion transportation included various types of ion transporters. For example, nhe1 is a cation transporter for H^+ and Na^+ ions, kcc is an anion/cation cotransporter for K^+ and Cl^-, and clic1 is a member of the Cl^- channel family of anion transporters. These genes can help balance osmosis between the inner cells and the environment. Our results showed that genes encoding water channel proteins were down-regulated in juveniles subjected to high-salinity treatment. We propose that the changes in expression patterns of osmoregulation-related genes play an important role in the physiological responses of juveniles to salinity changes. Ion transport-related genes can be divided into three categories: cation transporters (e.g., *NHE1*), anion/cation cotransporters (e.g., *kcc* and *glvr2*), and anion transporters (e.g., *pres*, *clic1*, and *slc26a6*). Our results showed that *Aqp 1* and *Aqp 11* were both down-regulated in juveniles under high-salinity conditions. Under high-salinity treatment, solute carrier family genes and water channel proteins were differentially expressed in juveniles, but not in embryos (Supplementary Figure S5). The gill and kidney are

vital osmoregulatory organs in fish. Histological analyses showed more goblet and chloride cells in the juveniles than in the embryos. In addition, pavement cells were only observed in juveniles. Both embryos and juveniles had aglomerular kidneys (Supplementary Figure S6) and both can respond to changes in salinity. Previous research has shown that ion transporter genes are highly expressed in the brood pouch of pregnant seahorses to maintain osmotic stability during embryonic development (Whittington et al., 2015). Therefore, parental care is vital for osmoregulation during embryonic development.

Cluster analysis generated nine clusters (Figure 1E). Four networks were identified based on related KEGG pathways: i.e., embryonic development, immune response, amino acid metabolism, and carbohydrate metabolism (Figure 1F). Hub genes, such as *cdk1*, *ccna2*, *cdc20*, *mcm6*, and *mcm3*, which are related to cell proliferation and DNA replication, were found in the protein-protein network (Figure 1G). This indicates that cell proliferation and DNA replication genes were differentially expressed between embryos and juveniles. Additional hub genes, including *ndufa6*, *ndufa10*, *ndufa12*, *cox7c*, *cox8a*, *atg5*, *atg13*, and *ctsd*, which are involved in metabolism and immune response, were also detected in this study (Figure 1G).

In summary, our study revealed that changes in salinity had a significant effect on the physiological processes of freshwater pipefish embryos and juveniles. Although parental care provided by the brood pouch was vital for embryonic development, salinity exposure still affected embryonic development in pipefish. Compared to embryos, juvenile pipefish exhibited marked changes in carbohydrate metabolism, ion transporters, and immune traits in response to saline environments, further highlighting the essential role of the brood pouch in protecting against environmental challenges and in driving evolutionary adaptation in freshwater pipefish.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

DATA AVAILABILITY

Raw data were deposited in the National Center for Biotechnology Information database under BioProjectID: PRJNA890450 and Sequence Read Archive under SRA accession No.: SRR21913252 to SRR21913266. Supplementary data were submitted to the Science Data Bank database (DOI: 10.57760/sciencedb.j00139.00050).

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

Q.L. and Y.H.Z. designed the study. S.X.F. conducted the experiment and performed data analysis. S.X.F. and Y.H.Z. drafted the paper. X.W., G.Q., and Y.Z. revised the paper. All authors read and approved the final version of the manuscript.

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