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Comparative analysis of phosphoproteomic in the intestine of *Sepia lycidas* under different salinity environments

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Abstract

Cuttlefish are sensitive to the breeding environment, and the low-salinity environment significantly impacts their growth and immunity. So far, it is difficult to breed this species artificially. This study was conducted in *Sepia lycidas.* And the aim was to investigate the differences in protein phosphorylation in the intestine of *S. lycidas* under different salinity conditions. Firstly, 999 phosphoproteins (specific peptide ≥ 1), 1928 phosphopeptides, and 2727 phosphorylation sites were identified. Among them were 284 down-regulated expression phosphorylation sites (corresponding to 115 phosphoproteins) and 674 up-regulated expression phosphorylation sites (corresponding to 408 phosphoproteins) in the intestine under a low salinity environment compared with that under a natural salinity environment. Next, GO analysis found that more phosphoproteins corresponding to differentially expressed phosphorylation sites were related to anatomical structure development, multicellular organism development, regulation of the cellular process, etc. The molecular functions of these proteins mainly contain protein binding, transferase activity, catalytic activity, and heterocyclic compound binding. And they are mainly involved in the cellular components of intracellular anatomical structure, organelle, and cytoplasm. KEGG enrichment analysis of the differential phosphoproteins suggested that many significantly enriched pathways were related to the phosphatidylinositol signaling system, cell junction (adherens junction and tight junction), and inositol phosphate metabolism. Finally, changes in environmental salinity can affect the intestinal structure, metabolism, and immune homeostasis of *S. lycidas*.

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Introduction

Sepia lycidas is a benthic species belonging to Mollusca, Cephalopoda, Coleoidea, sepiada, Spiidae, and Sepia. This species is also well known as a marine resource because of its high nutritional value and artificial breeding prospects in Southeast Asia. Recently, some studies have been carried out on several aspects of *S. lycidas*. For example, Murata et al. analyzed the role of neuroendocrine regulators in gonadal sex differentiation during the reproductive process of *S. lycidas* (Murata et al. 2021); Peng et al. investigated the effect of light intensity on the embryo development of *S. lycidas,* indicating that different light conditions should be provided throughout the embryonic developmental stages based on their sensitivity to light (Peng et al. 2019); Lucky et al. studied the behavioral laterality of *S. lycidas* and found that they exhibit behavioral dimorphism and morphological antisymmetric in natural populations (Lucky et al. 2012); Han et al. have revealed that a 9.56% fat content in the diets might be the optimum content for maintaining the growth and normal metabolism of juvenile *S. lycidas* (Han et al. 2017).

Normally, the squid is sensitive to environmental changes, and the breeding environment could primarily affect the growth and survival rates. As one of aquaculture's most critical environmental factors, salinity could significantly affect aquatic organisms' growth and various physiological activities, including osmoregulation, metabolism, and reproductive activities (Kültz 2015; Lee et al. 2022). However, limited is known on the effect of salinity on squid.

The intestine is the leading site of nutrient digestion and absorption and one of the largest immune organs in vertebrates. Complete morphological structures could maintain the normal function of the intestine. Studies on aquatic animals have revealed that changes in salinity could cause damage to the characteristics of intestinal histomorphological structures, affect the function of the epithelial cells involved in nutrient digestion and osmoregulation, and alter the intestinal microbial composition, which could also affect immunity (Luo et al. 2021). Therefore, the intestine as the research object is significantly beneficial to research squid artificial culture.

Protein phosphorylation, one of the post-translational modifications (PTM) processes, is the reversible process by which proteins transfer phosphate groups on adenosine triphosphate (ATP) or guanosine triphosphate (GTP) to serine (Ser), threonine (Thr) or tyrosine (Tyr) residues, catalyzed by protein kinases (Garcia-Garcia et al. 2016). Phosphorylation is a rapid and reversible enzymatic reaction that regulates protein activity and cellular processes through the attachment and detachment of phosphodiester groups on protein residues (Cohen 2002; Mi et al. 2021). Therefore, this process has participated in almost all the processes of cells, such as metabolism, development and differentiation, apoptosis, signal transduction, cell movement, and tumorigenesis (Ardito et al. 2017; Garcia-Garcia et al. 2016). From these, the study of intestinal phosphorylation is of great significance for studying the series of regulatory responses of squid in response to environmental changes.

There are few studies on *S. lycidas*, especially on the effect of salinity on its physiological and molecular regulation. From the proteomics perspective, this study aims to understand the effects of different growth environments, especially water salinity, on the intestine of *S. lycidas* and provide a theoretical basis for the subsequent work on the physiology and breeding of *S. lycidas*.

Materials and Methods

Experimental materials

S. lycidas were obtained from Donghai Island (Zhanjiang, China). Intestinal samples of three *S. lycidas* under the natural seawater environment (30‰) were collected and mixed together in equal as control; intestinal samples of three individuals under the low-salinity environment (24±1‰) were collected and mixed as the experimental sample. After being washed with sterilized saline, the samples were frozen at -80℃ for later use.

Experimental methods

Extraction and collection of sample proteins

1) Grind the intestinal samples into powder, and add Lysis buffer (8 M Urea, 50mM NH_4HCO_3, 1 mM sodium orthovanadate, protease phosphatase inhibitor) to extract the protein;

2) Keep on ice for 30 minutes, then centrifuge at 13,000 rpm for 15 minutes at 4 °C, collecting the supernatant, and store in a -80 °C refrigerator;

3) The extracted proteins were detected whether degradation and equal by SDS-PAGE. Total protein concentration was measured using the Bradford method.

4) *Trypsin digestion and enrichment of phosphopeptides*

The protein samples were digested using Trypsin in reference to the ultrafiltration tube enzyme digestion method (Wisniewski et al. 2009). After digestion, the peptides were enriched using High-Select™ TiO_2 Phosphopeptide Enrichment Kit (Thermo Fisher). The enrichment method was carried out according to kit instructions.

LC-MS/MS Analysis

The peptide was re-dissolved in 0.1 % formic acid solution. Then, the peptide solution was separated by EASY-nLC liquid phase and low pH reverse-phase C18 capillary chromatography (150 μm×150 mm, 1.9 μm). Using a high-sensitivity mode, the Orbitrap Exploris 480 mass spectrometer was employed to identify the peptide solution. Each full scan was a high-speed signal-dependent scan with a scanning time of 105 min. The first-level full scan resolution was 60000, the scan range was 350-1400 m/z, and the maximum injection time was 22 ms. The collision energy was 28 %, secondary scan resolution was 15000, charge state screening (containing precursors with +2 to +6 charges), maximum injection time was 22 ms, and dynamic elimination was 30 s.

Data comparison and analysis

In this experiment, the obtained raw data were blasted in the reference database by MaxQuant (version 2.0.1.0) software for qualitative and quantitative identification of proteins and peptides and statistically analyzed to obtain phosphopeptides (Rigbolt et al. 2011). The reference database was the *Sepia pharaonis* database: GCA_903632075. 3 spha2. 0.

The absolute value of the ratio of the quantitative protein data of the two groups of samples is more significant than twice the criterion for the selection of differential peptides. Statistical significance was accepted at P value≤0.05.

OmicsBox (version: 2.0.10) annotated the differentially expressed phosphoproteins. All functional information related to these differentially expressed phosphoproteins was obtained, including Gene Ontology (GO) and KEGG pathway information.

Motif analysis: Using the Position weight matrix (PWM) scores algorithm, motif analysis was performed on the phosphorylation sites expressed in relative abundance in cells.

Results

Phosphorylated peptide quantification

A total of 999 phosphoproteins (specific peptide ≥ 1), 1928 phosphopeptides, and 2727 phosphorylation sites (including 1914 class1 phosphorylation sites) were identified by LC-MS analysis. Phosphorylation enrichment efficiency was about 88%. As shown in **Table 1**.

Among them, the phosphorylation reactions are mainly concentrated on amino acid residues such as serine (S), threonine (T), and tyrosine (Y), and the phosphorylation ratios in the three amino acid residues are pretty different. As shown in **Figure 1**, serine has the most modification sites among the three amino acids, with 1713 modification sites, followed by threonine, with 157 modification sites, and tyrosine modification sites only 44. For most

phosphoproteins, there is usually only one phosphorylation modification site on the sequence, but there are also a few proteins with multiple site modifications—**Figures 1** and **2**.

Table 1 Basic identification information of Quantitative phosphoproteomic

Item	*number*
phosphoproteins	999
Phosphopeptides	1928
phosphorylation sites	2727
Class 1 phosphorylation sites	1914

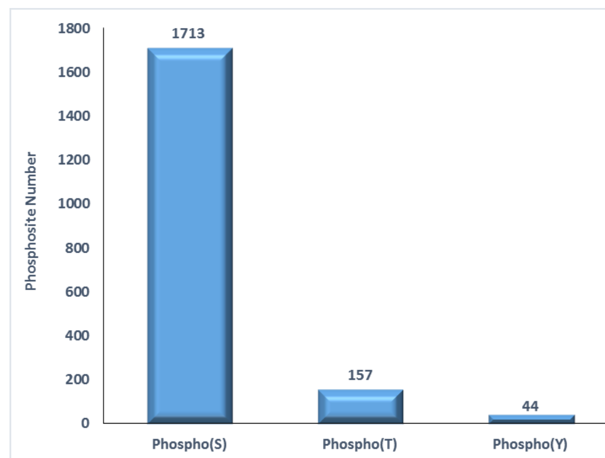

Figure 1 Statistics on the number of Serine(S), Threonine(T), Tyrosine(Y) phosphorylation sites

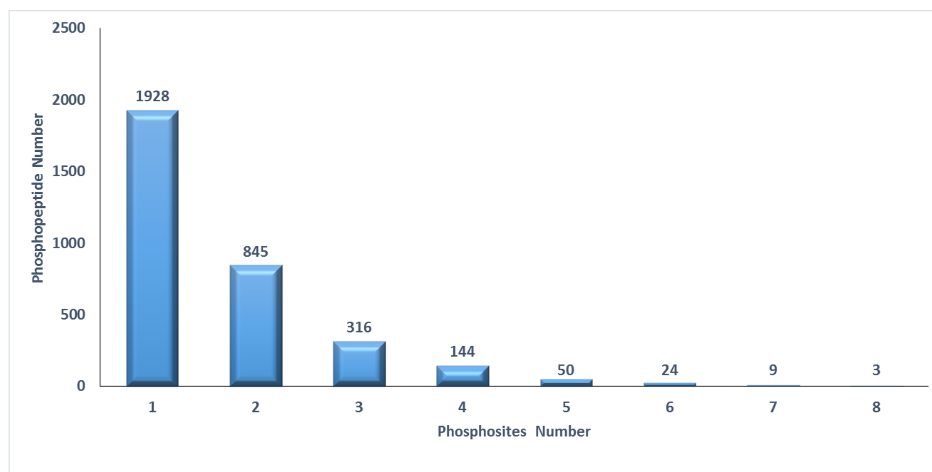

Figure 2 Statistics of the number of sites on the identified phosphopeptides

Screening of differentially phosphorylated sites

Compared with the control groups, 958 phosphorylated sites with significant differences were screened, corresponding to 523 phosphoproteins. Among them, 284 phosphorylated sites were down-regulated expression, and 674 were up-regulated.

Table 2 Statistical results of differential proteins from *S. lycidas*

item	*number*
Significantly different phosphorylated sites	958
down-regulated phosphorylated sites (corresponding to phosphoproteins)	284(115)
up-regulated phosphorylated sites (corresponding to phosphoproteins)	674(408)

GO classification analysis

GO classification analysis was performed for differential expression phosphorylated sites corresponding to phosphoproteins (differentially expression phosphoproteins, DEPPs) (the first 20 terms of the three classifications were plotted). As shown in **Figure 3**, these DEPPs are mainly involved in the anatomical structure development, multicellular organism development, regulation of the cellular process, cellular component organization, cellular metabolic process, and other biological processes; protein binding, transferase activity, catalytic activity, acting on a protein and other molecular function; intracellular anatomical structure, organelle, cytoplasm, and other cellular components.

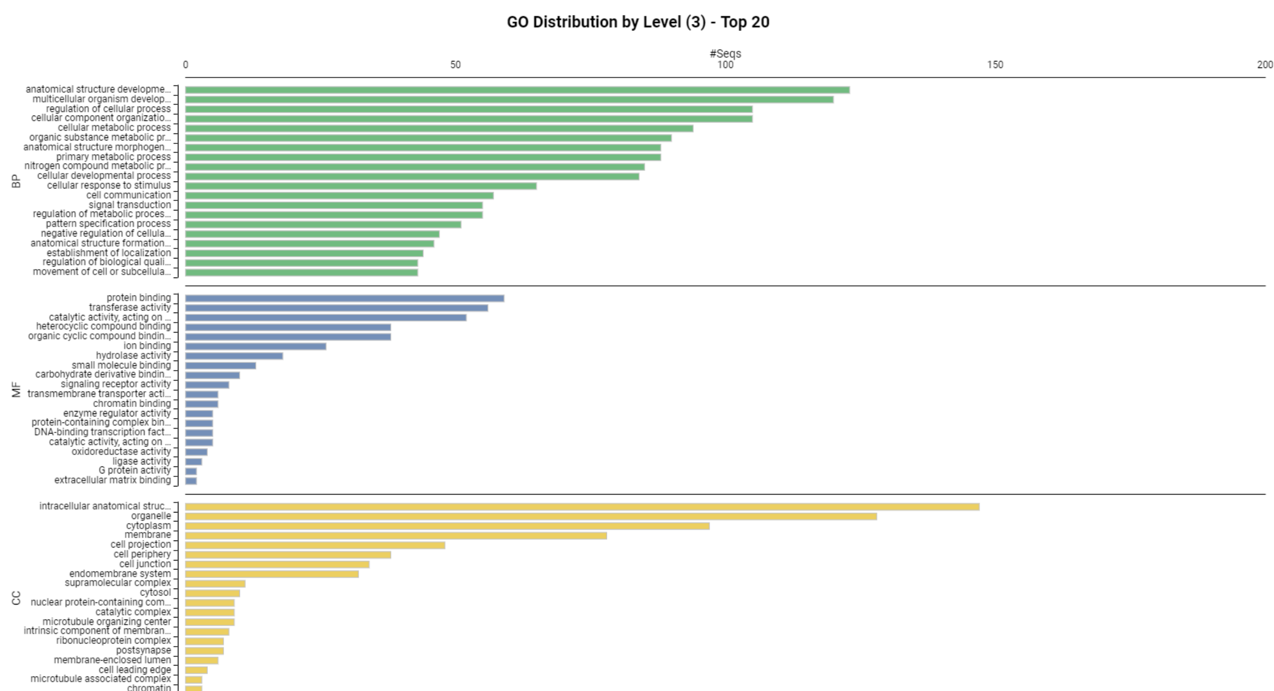

Figure 3 GO analysis histogram of DEPPs**,** GO term: BP: biological processes; MF: molecular function; CC: cellular component

KEGG enrichment analysis

The distribution of DEPPs under the top 20 functional pathways was screened according to the *P*-value, as shown in **Figure 4**. It was found that they were mainly involved in the Phosphatidylinositol signaling system, Cell junctions (Adherens junction and Tight junction), Inositol phosphate metabolism, and so on.

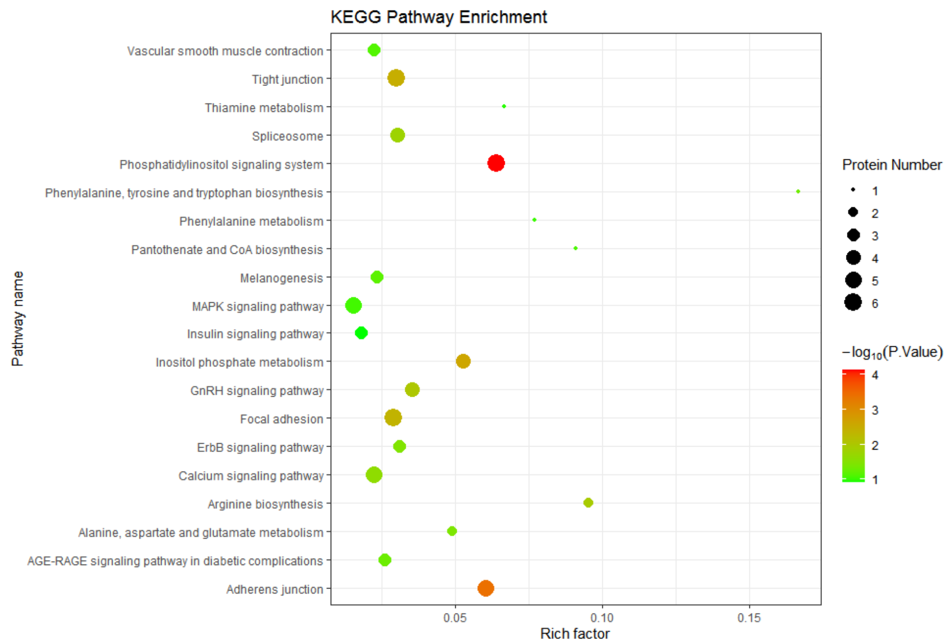

Figure 4 KEGG pathway enrichment analysis bubble chart of DEPPs.

Motif analysis of differential phosphorylation sites

The analysis used a peptide sequence with 15 amino acids centered on the modification site. Serine (S) and threonine (T) were used as the center modification sites to analyze, respectively. As shown in **Figure 5**. The results showed that the Motif structure of the serine (S) phosphorylation site was inconsistent. The Motif of threonine (T) as the central modification site had more proteins with xTPx motifs.

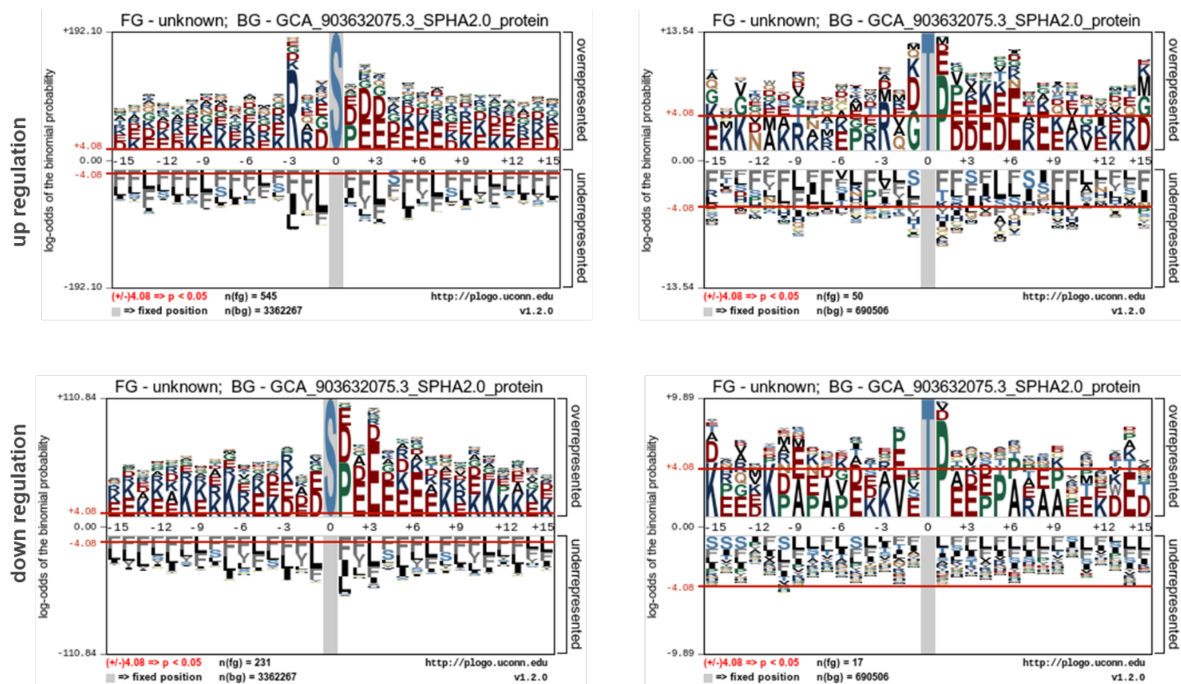

Figure 5 Motif analysis of phosphoproteins**.** Left were the statistics of the motif structure of the serine (S) phosphorylation site; right were the statistics of the motif structure of the threonine (T) phosphorylation site.

Discussion

The intestine is a complex organ with multiple physiological functions. Its primary function is digestion and absorption, allowing nutrients to pass from the intestinal cavity into the circulatory system and the internal environment of the body; and the intestine is also vital in barrier protection of the body, which can prevent substances invasions such as pathogenic microorganisms, and proinflammatory cytokines (Farhadi et al. 2003). Numerous studies have shown that salinity stress can significantly affect intestinal structure, digestive enzyme activity, antioxidant capacity, non-specific immune function, and the composition of the intestinal microbiota of aquatic animals. Salinity changes can directly affect aquatic animals' growth, energy metabolism, stress resistance, and disease resistance, and many genes will respond significantly to salinity stress (Paital and Chainy, 2010; Xie et al., 2019).

In this study, we analyzed the DEPPs in the intestinal of *S. lycidas*. After GO analysis, we found that these DEPPs were mainly involved in biological processes such as multicellular organism development and substance metabolic processes. Among the molecular functions, it is more concentrated in protein binding, transferase activity, catalytic activity, and heterocyclic compound binding. In previous studies on the effect of salinity on aquatic animals, many research results showed that proteins related to osmotic pressure regulation, ion transport mechanisms, and other biological processes played an important role (Ruiz-Jarabo et al. 2017; Schauer et al. 2018), followed by some material metabolism-related processes (Xiong et al. 2020). In this study, the GO results of DEPPs were similar to those of these studies, such as more proteins in the biological process associated with the substance metabolic process. However, there were some differences, for example, in multicellular organism development. Many studies focus on transcripts or proteome analysis. In contrast, our study focuses on the GO analysis of DEPPs, which is essential in regulating functions. However, in molecular functions, the functions of DEPPs are more involved such as protein binding, transferase activity, catalytic activity, and heterocyclic compound binding. Protein binding often occurs in many regulatory processes, such as protein interaction, protein and nucleic acid interaction,

and signal transduction. Transferases are enzymes that catalyze the transfer or exchange of certain groups (such as acetyl, methyl, amino, phosphate, etc.) between substrates, which dominate many catalytic processes such as metabolism, nutrition, and energy conversion. Our study found dozens of DEPPs classified as transferases, including aminotransferase, Ubiquitin transferase, protein kinase, ribosyltransferase, etc. Similarly, the study has shown that Nicotinamide phosphoribosyltransferase (Nampt) of hybrid crucian carp significantly reduced intestinal permeability and apoptosis, protected the intestinal barrier, and enhanced host immune defense against bacterial infection (Tang et al. 2022). Joana Abrantes et al. (Abrantes et al. 2009) found fucosyl transferase (Fut2) mediated a variety of susceptible mutant pathogens, which play an important role in the protection of mammalian gastrointestinal tract. In this study, GO analysis showed that in response to salinity changes, *S. Lycidas* started a series of regulatory effects, not only affects the development and structure of the intestine, but also may affect the metabolism and immune function of *S. lycidas*.

In addition, after KEGG enrichment analysis, it was found that the significantly DEPPs were closely related to phosphatidylinositol signaling system, cell connections (such as adherens junction, tight junction), and inositol phosphate metabolism pathways.

Cell junction is the specialized junction structure formed between adjacent cells or between cells and extracellular matrix in the plasma membrane contact area, including adhesion connection and tight connection. It strengthens the mechanical connection between cells and maintains the integrity and coordination of tissue structure. Adhesion connections initiate cell-cell contact and mediate the maturation and maintenance of contact; the tight junction regulates the intercellular ion and solute movement of the paracellular pathway (Hartsock & Nelson 2008).

Adhesion junction is usually located below the tight junction, which is a large complex connecting and mediates the mechanical connection between adjacent cells. It ensures the direct contact between cells and plays an indispensable role in maintaining the tissue structure (Van Campenhout et al. 2019). Its main functions include starting up and stabilizing cell-cell adhesion, regulating actin cytoskeleton, intracellular signal transduction and transcriptional regulation. The formation, maintenance and related functions of adhesive junctions are essential for maintaining intestinal morphology and homeostasis (Chen et al. 2021). Studies have shown that squid ink polysaccharide can protect the tight and adhesion junction of intestinal epithelial cells in animals by increasing the expression of proteins related tight junction and adhesion junction, thereby protecting the intestinal barrier from damage (infection) (Zuo et al. 2015). Tight junction is the main connection mode between intestinal epithelial cells, which regulates the information transmission between inside and outside cells and plays an important role in regulating intestinal barrier function and permeability (Suzuki 2013). Intestinal health is related to intestinal structural integrity, which is linked to proteins associated with tight junctions. Tight junction-related proteins are essential molecules that maintain intestinal barrier function and determine intestinal wall permeability (Rodriguez-Feo et al. 2015). It is reported that high-fat diet (HFD) significantly inhibits the mRNA expression of epithelial tight junction proteins (ZO-1, occludin and claudin-3), resulting in increased intestinal permeability of juvenile grass carp (Liu et al. 2022). In this study, the KEGG of DEPPs of *S. lycidas* under different salinity showed that the proteins of adhesion and tight junction-related genes were changed, indicating that they played an essential role in *S. lycidas* under low salinity stress. It was revealed that too low salinity might cause changes in adhesion and tight junction proteins of intestinal epithelial cells, destroying the intestinal barrier function of *S. lycidas*, thereby affecting the average growth of *S. lycidas*. Phosphatidylinositol (PI) is the essential component of inositol lipids in eukaryotic cells. Different types of PI molecules in cells convert to each other under specific phosphatidylinositol kinase and phosphatase. PI is a low abundance but essential biological function phospholipid distributed in various eukaryotic biofilms. PI derivatives are important secondary messengers that regulate various cellular processes, including proliferation, cytoskeleton, and material

transport (Boldyreva et al. 2021). The phosphatidylinositol signaling pathway is the critical signal transduction pathway responsible for producing many receptor-mediated cellular responses (Hale et al., 2020). For example, in the study of inflammatory bowel disease, it was confirmed that phosphoinositide 3-kinase, which can phosphorylate phosphatidylinositol plays an essential role in maintaining mucosal homeostasis (Zhao et al. 2018). Similarly, in this study, DEPPs were mainly enriched in phosphatidylinositol signaling and metabolic pathways at different salinities, indicating that phosphatidylinositol plays a crucial role in intestinal homeostasis.

In addition, the Motif analysis of different phosphorylation sites showed that the structure of the Motif was more diverse, which may be due to the extensive influence of different salinity on various aspects of squid, and involving more differentially phosphorylated proteins.

In conclusion, through the comparative analysis of proteins phosphorylation in the intestinal of *S. lycidas*, it was found that the growth and development of *S. lycidas* was sensitive to the environment of different salinity, and the structural integrity and health of the intestinal were significantly affected.

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