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Identification of free amino acids (FAA) that are important as major intracellular osmolytes in the estuarine Hong Kong oyster, *Crassostrea hongkongensis*

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ABSTRACT

Crassostrea hongkongensis (Hong Kong oyster), a commercially valuable bivalve thriving in the estuaries along the northern coast of the South China Sea, is threatened by the increase of salinity during the dry season. It is essential to improve its hyper-salinity tolerance, which requires a clear understanding of the mechanism to regulate its osmotic balance. For this purpose, 25 free amino acids (FAA), the most critical intracellular osmolyte in bivalves, were quantified in gill, adductor muscle, and hemolymph of *C. hongkongensis* during abrupt salinity changes (18–6, 12, 24 and 30‰) by high-performance liquid chromatography-tandem mass spectrometry. Taurine, alanine and glycine were dominant FAA, contributing to total FAA contents that varied with salinities, no matter in gill, muscle or hemolymph. Under hypo-osmotic stress, total FAA contents decreased in gill and adductor muscle but increased in hemolymph, probably resulting from the extrusion of some FAA from tissues to prevent swelling of cells. And taurine contributed most significantly to the hypo-salinity response, which was mainly reflected by the remarkable increase in hemolymph at 8 h after the exposure to 6‰. By contrast, total FAA contents increased in gill, muscle, and hemolymph when transferred to hyper-salinity, suggesting the assumption that it increases the osmolality and subsequently prevents the cell shrinking. Alanine and glycine contributed most significantly to hyper-salinity response. Of the two, the former was more sensitive to hyper-osmotic stress for its rapid increase in gill within 8 h after the exposure to 24 and 30‰. An inverse pattern with most FAA was presented by ornithine, the intermediate of the urea cycle, which was probably associated with the FAA metabolism. This study identified the major FAA as intracellular osmolytes in *C. hongkongensis* and would benefit the further understanding of the regulatory mechanisms of oyster salinity acclimation.

1. Introduction

The Hong Kong oyster, *Crassostrea hongkongensis*, is a commercially valuable aquaculture species mainly distributed along the northern coast of the South China Sea (Lam and Morton, 2004). It can tolerate salinity ranging from 5 to 30 ppt and typically thrives within a salinity range of 10–25 ppt (Xiao et al., 2018; She et al., 2022). Because of its low resistance to high salinity, the cultivation is limited to estuaries and is usually accompanied by seasonal transfer among estuarine aquafarms where salinity changes dramatically with the season (She et al., 2022). Also, in recent years, less rain and surface fresh water that might be related to climate change in South China has caused a violent increase in salinity in estuarine regions, leading to the mass death of *C. hongkongensis* (Qin et al., 2021). Hence, there is an urgent need for a new strain with hyper-salinity tolerance, which requires a profound understanding of the regulatory mechanism of *C. hongkongensis* salinity

Abbreviation: FAA, free amino acids; HPLC-MS/MS, high-performance liquid chromatography tandem mass spectrometry; Tau, taurine; Ala, alanine; Gly, glycine; Glu, glutamic acid; Orn, ornithine; Asp, aspartic acid; Pro, proline; β-Ala, β-alanine; Lys, lysine; Gln, glutamine; Asn, asparagine; Arg, arginine.

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acclimation.

The fluctuating salinity in estuaries presents a great osmotic challenge to marine invertebrates, and for osmoconforming species, the osmolyte system plays an essential role at a cellular level in response to osmotic stress (Pierce and Amende, 1981; Hosoi et al., 2005). In this process, free amino acids (FAA) have been demonstrated to contribute dominantly to intracellular osmolality and cell volume regulation (McNamara et al., 2004; Rivera-Ingraham and Lignot, 2017; Pourmozaffar et al., 2020). Measurements of FAA concentrations of some crustaceans showed that total FAA pools contribute 10–20 % to intracellular osmolarities during salinity acclimation, and glycine, proline, taurine and alanine were the most common osmolytes (McNamara et al., 2004; Rivera-Ingraham and Lignot, 2017; Koyama et al., 2018). A more important osmotic effect of FAA was found in several bivalve mollusks (20–30 % of the total osmotically active substances), and differences in osmoregulatory FAA are observed among species (Henry and Mangum, 1980; Huo et al., 2014; Pourmozaffar et al., 2020). For example, glycine was speculated as one of the most essential osmolyte in the mussel *Mya arenaria*, while alanine might be the major osmoregulatory FAA in *Corbicula japonica* and *Macoma balthica* (Matsushima et al., 1984; Sokolowski et al., 2019; Haider et al., 2019).

Studies on FAA as osmolytes in oysters have been performed since the 1960s Lynch and Wood (1966) analyzed FAA concentration in the adductor muscle of *Crassostrea virginica* for the first time and found that taurine, alanine, glycine, and proline changed significantly with salinity. FAA in the mantle of *C. virginica* was identified later, supporting the contribution of taurine, alanine, aspartic acid, and glycine in osmotic adjustment (Heavers and Hammen, 1985). An investigation of *Crassostrea gigas* exposed to abrupt salinity change indicated that alanine might be essential to short-term response to hyperosmolality, while taurine showed a slower and substantial increase that contributes to a long-term adaptation to hyper-osmolality (Hosoi et al., 2003). Further, taurine-related genes in *C. gigas*, such as cysteine sulfinate decarboxylase and taurine transporter, were explored to understand the regulation mechanism of FAA contents in response to osmotic stress (Hosoi et al., 2007; Meng et al., 2013; Zhao et al., 2017), with the hope of gaining candidate genes for genetic improvement of salinity tolerance. However, the above studies have two main limitations: a small number of FAA (seven or eight kinds for each analyzation) and a single type of tissue (muscle, mantle or gill analyzed solely in most the cases). Until now, a complete picture of the FAA change from hemolymph to the intracellular fluid of oysters in different salinities is not clear.

Although the gill transcriptome sequencing has revealed a hint that FAA is involved in *C. hongkongensis* salinity acclimation (Zhao et al., 2014; Xiao et al., 2018; She et al., 2022), there is still limited knowledge about the FAA osmolyte system and the main contributor to osmotic regulation. For marine invertebrates, hemolymph was the extra-cellular fluid with abundant FAA as an osmolyte (Pierce, 1982; Fang et al., 1992). Moreover, gill and muscle were speculated as the main sites for metabolism and accumulation of FAA during salinity adaption (Meng et al., 2013; Lin et al., 2016; Nagasaki et al., 2018). Hence, we measured 25 FAAs in the gill, adductor muscle, and hemolymph of *C. hongkongensis* exposed to abrupt changes in salinity based on high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS). This work aims to create an accurate global view of FAA osmolyte system for studying the mechanisms of intracellular osmoregulation in *C. hongkongensis*. Further, identifying major FAA as osmolyte may provide the research direction of the candidate genes for salinity tolerance, in the hope of contributing to genetic improvement.

2. Materials and methods

2.1. Animal treatment and sample collection

The Hong Kong oyster is not an endangered or protected species, and no specific permits were required for our studies. *C. hongkongensis* at two years old (shell length 64.26 ± 7.18 mm) were collected from a local oyster farm (salinity 16–20‰) in Zhanjiang, Guangdong province, China, in April 2022. Part of the shell edges (about 10 mm long and 5 mm wide) of each oyster was chipped away to ensure the free exchange of seawater between the inside and outside of the shell. Then oysters were acclimatized over a week in 18‰ filtered sea water (FSW). After acclimation, some oysters were maintained in 18‰ FSW as control, and others were transferred to 6‰, 12‰, 24‰ and 30‰ FSW, respectively. Each treatment consisted of three replicates with 15 oysters per replicate. During the experiment, the oysters were not fed.

Two specimens were taken randomly for per replication tank at 8, 24, 48, and 72 h after transfer. And for the control group, an additional six were randomly taken at 0 h. Hemolymph was taken from the pericardial space through the notch of shell with a syringe. Then the gills and adductor muscles were dissected from oysters immediately. Samples were frozen under liquid nitrogen and stored at − 80 °C. The gill, adductor muscle, and hemolymph samples of one oyster per replicate were selected, and finally 189 samples from 63 oysters were used for subsequent FAA measurements.

2.2. Chemical standards and derivatization procedure

Twenty-five amino acids (Aladdin, China) were used as standards, and the standard chemicals mixture information was provided in Table S1. Gill and adductor muscle samples of each oyster were individually weighted and homogenized in distilled water, which was detailed in Table S2. Fifty microliters of protein precipitation regent (Beijing Mass Spectrometry Medical Research Co., Ltd., China) was added to a 50 μl sample solution. After vortex mixing, it was centrifuged at 13,200 rpm for 4 min at 4 °C. Ten microliters supernatant was mixed with 50 μl of borate buffer (0.1 M, pH 8.8), and the solution was derivatized with 20 μl AQC Reagent (Waters, USA) at 55 °C for 15 min. Samples were chilled on ice and centrifuged shortly for FAA analysis.

2.3. High-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS)

Amino acids composition was determined by high-performance liquid chromatography (Ultimate 3000, USA) tandem mass spectrometry (API 3200 Q TRAP, USA) (HPLC-MS/MS). Samples were transferred to insert vials and 50 μl were injected into the HPLC. Chromatographic separations were performed on an MSLab50AA-C18 column (150 × 4.6 mm, 5 μm). The mobile phase consisted of water with 1‰ formic acid (A) and acetonitrile with 1‰ formic acid (B). The solvent was delivered to the column at a flow rate of 1 ml min^{-1} with the column temperature of 50 °C as follows: 0–1 min from A-B (95:5) to A-B (95:5); 1–1.1 min from A-B (95:5) to A-B (50:50); 1.1–12 min from A-B (50:50) to A-B (30:70); 12–12.1 min from A-B (30:70) to A-B (0:100); 12.1–15 min from A-B (0:100) to A-B (0:100); 15–15.1 min from A-B (0:100) to A-B (95:5); 15.1–20 min from A-B (95:5) to A-B (95:5). The parameters for MS-MS detection were as follows: mode: positive-ion mode; ion spray voltage: 5500 V; nebulizer gas pressure: 55 psi; curtain gas pressure: 20 psi; collision gas pressure: medium; turbo gas temperature: 500 °C; entrance potential: 10 V; collision cell exit potential: 2 V. And nitrogen gas was used as the collision gas in the multiple reactions monitoring mode. The data was obtained using Analyst software version 1.5.1 (Applied Biosystem, USA).

2.4. Statistical analysis

All data analyses were performed using Graphpad Prism 9.0 and IBM SPSS Statistics 26.0. Statistical significance analysis was performed by Duncan's multiple range test for samples from different groups at the same time or these from the same group at different time. FAA concentrations in the same tissue samples from 5 groups at different time were also evaluated using two-way analysis of variance (Two-way

ANOVA). $P < 0.05$ was used as the level of significance. The contribution of each type of FAA to salinity response was clarified as follows:

$$\text{Contribnution}(\%) = \frac{\text{FAA Contents}_{mn} - \text{FAA Contents}_{cn}}{\text{TotalFAA Contents}_{mn} - \text{TotalFAA Contents}_{cn}} * 100\%$$

m: treatment group.

n: sampling time.

c: control group (18‰ FSW).

Principle component analysis (PCA) was performed to identify the major free amino acids in response to salinity changes. Eigenvalue > 1 was used for the identification of significant principal components (PCs), and factor loading > 0.60 were used for the identification of main factors in PCs (Granato et al., 2018).

3. Results

3.1. FAA compositions of gill, adductor muscle, and hemolymph from oysters in the control group

Twenty-five FAA contents in gill, adductor muscle, and hemolymph from 63 oysters in different salinity levels were detailed in Table S3-S5, respectively. In the control group (18‰ FSW), the total FAA in the gill of oysters was 56.76–62.54 μmol/g through the duration of the experiment (Table 1). Taurine (Tau), alanine (Ala), glycine (Gly), glutamic acid (Glu), ornithine (Orn) and aspartic acid (Asp) were the dominant FAA (> 5 % of total contents), accounting for 81.63–87.68 % of the full content (Table 2). Meanwhile, proline (Pro), β-alanine (β-Ala), Lysine (Lys), glutamine (Gln), and asparagine (Asn) were the major components (1–3 % of total contents) in the remaining (Table S3). Total FAA contents in the adductor muscle of oysters were 46.79–67.15 μmol/g through the duration of the experiment (Table 1). Tau, Ala, Gly, Glu, and Orn were also abundant in the muscle, accounting for 76.97–85.21 % of the total contents of FAA (Table 2), and Asp, Pro, β-Ala and Arginine (Arg) were the major components in the remaining (Table S4). FAA contents in the

Table 1
Total FAA contents in muscle, gill and hemolymph from oysters in different salinity levels.

Salinity (‰)	Time (h)	Total FAA concentrations		
		Gill (μmol/g)	Muscle (μmol/g)	Hemolymph (μmol/L)
6	8	37.76 ± 9.30	34.68 ± 3.09*	3395.24 ± 2230.82†
	24	40.70 ± 7.19	23.53 ± 1.60*	1566.74 ± 747.84
	48	28.74 ± 4.84**	17.44 ± 3.88*	1435.67 ± 527.24
	72	46.55 ± 7.67	20.15 ± 2.49*	1595.70 ± 289.94†
12	8	41.59 ± 5.30	47.48 ± 5.94	3090.76 ± 212.83†
	24	53.63 ± 14.59	39.90 ± 1.26	2779.43 ± 645.22†
	48	50.16 ± 6.72	37.65 ± 8.93	2627.06 ± 291.29†
	72	67.34 ± 20.62	45.63 ± 1.89	1912.45 ± 162.07†
18	0	56.76 ± 2.54	67.15 ± 9.17	2363.28 ± 211.09
	8	59.60 ± 11.28	56.77 ± 8.51	1253.44 ± 159.60
	24	59.14 ± 14.60	46.79 ± 5.44	1510.941 ± 24.57
	48	62.54 ± 7.90	49.78 ± 17.48	1052.334 ± 67.41
	72	61.89 ± 18.86	52.14 ± 10.59	850.28 ± 96.02
24	8	89.47 ± 10.27	79.58 ± 22.13	2977.86 ± 309.81†
	24	79.36 ± 11.37	68.39 ± 5.48*	2103.00 ± 61.24†
	48	74.37 ± 12.877	55.69 ± 1.67	1275.52 ± 55.37†
	72	87.26 ± 9.31	85.41 ± 14.47*	1787.58 ± 68.04†
30	8	75.01 ± 4.13	52.54 ± 17.95	2004.50 ± 133.46†
	24	63.99 ± 8.95	67.80 ± 15.47	1838.69 ± 204.21†
	48	115.20 ± 31.20**	81.71 ± 10.75*	1663.99 ± 49.978†
	72	111.38 ± 21.61**	108.83 ± 2.92*	2747.17 ± 150.19†

Values represent the mean value with standard deviation in three different individuals. Significant differences from the value in control group at the same time point are represented by *, ** and † in muscle, gill and hemolymph, respectively ($P < 0.05$).

Table 2
Predominant FAA in muscle, gill and hemolymph from oysters in 18 % FSW.

FAA	Time (h)	Concentrations and percentage		
		Gill (μmol/g)	Muscle (μmol/g)	Hemolymph (μmol/L)
Taurine (Tau)	0	20.38 ± 2.32 (35.95 %)	15.31 ± 1.27 (22.93 %)	442.95 ± 127.84 (18.56 %)
	8	21.30 ± 4.94 (35.97 %)	14.40 ± 3.33 (25.66 %)	316.92 ± 149.52 (24.54 %)
	24	23.63 ± 5.24 (40.11 %)	14.75 ± 1.71 (31.82 %)	461.22 ± 84.50 (30.47 %)
	48	26.28 ± 4.38 (41.91 %)	12.64 ± 5.04 (25.00 %)	235.04 ± 68.86 (22.13 %)
	72	19.49 ± 7.55 (31.24 %)	13.92 ± 3.32 (28.33 %)	169.49 ± 73.47 (19.48 %)
Alanine (Ala)	0	6.33 ± 2.38 (11.06 %)	15.13 ± 7.54 (21.97 %)	379.23 ± 206.86 (15.73 %)
	8	5.31 ± 0.88 (8.95 %)	7.55 ± 2.77 (13.95 %)	157.04 ± 59.33 (12.35 %)
	24	6.06 ± 1.86 (10.15 %)	8.41 ± 1.80 (17.84 %)	228.90 ± 84.12 (15.13 %)
	48	5.08 ± 0.43 (8.20 %)	6.29 ± 1.98 (12.82 %)	203.34 ± 46.68 (19.51 %)
	72	4.79 ± 1.44 (8.20 %)	5.58 ± 2.07 (10.56 %)	230.27 ± 78.51 (27.65 %)
Glycine (Gly)	0	7.51 ± 0.14 (13.26 %)	9.17 ± 6.55 (14.32 %)	179.72 ± 129.84 (7.79 %)
	8	8.42 ± 3.17 (13.82 %)	14.16 ± 9.94 (23.95 %)	169.06 ± 99.19 (14.30 %)
	24	3.64 ± 1.51 (6.17 %)	2.86 ± 0.64 (6.15 %)	121.72 ± 38.09 (8.08 %)
	48	6.95 ± 1.39 (11.34 %)	13.82 ± 6.50 (27.14 %)	157.43 ± 7.75 (14.98 %)
	72	11.23 ± 7.02 (17.13 %)	16.69 ± 11.64 (29.89 %)	32.56 ± 14.98 (3.74 %)
Glutamic acid (Glu)	0	6.44 ± 0.82 (11.33 %)	8.18 ± 3.66 (11.87 %)	66.46 ± 15.67 (2.79 %)
	8	5.47 ± 2.21 (8.93 %)	6.19 ± 0.92 (11.19 %)	111.13 ± 87.43 (8.44 %)
	24	7.14 ± 3.09 (11.77 %)	6.92 ± 3.60 (14.35 %)	118.05 ± 44.41 (7.78 %)
	48	7.10 ± 2.58 (11.11 %)	5.11 ± 2.55 (9.79 %)	43.45 ± 34.75 (4.16 %)
	72	5.40 ± 2.46 (8.55 %)	4.74 ± 0.45 (9.43 %)	34.12 ± 9.57 (3.99 %)
Ornithine (Orn)	0	4.55 ± 0.26 (8.03 %)	3.86 ± 1.07 (5.88 %)	252.46 ± 34.90 (10.66 %)
	8	4.51 ± 1.00 (7.80 %)	4.14 ± 1.45 (7.18 %)	59.12 ± 38.96 (4.75 %)
	24	5.98 ± 0.86 (10.27 %)	4.45 ± 0.18 (9.57 %)	97.38 ± 43.41 (6.47 %)
	48	5.42 ± 0.78 (8.67%)	4.48 ± 0.82 (9.46 %)	90.62 ± 13.35 (8.62 %)
	72	7.52 ± 4.16 (13.00 %)	3.60 ± 0.41 (7.00 %)	45.64 ± 22.76 (5.23 %)
Aspartic acid (Asp)	0	3.40 ± 0.23 (6.00 %)	2.60 ± 2.02 (3.72 %)	223.28 ± 38.38 (9.41 %)
	8	3.60 ± 0.66 (6.16 %)	0.34 ± 0.44 (0.63 %)	98.39 ± 37.95 (7.98 %)
	24	3.71 ± 1.61 (6.16 %)	0.65 ± 0.48 (1.32 %)	107.66 ± 45.90 (7.14 %)
	48	4.09 ± 1.31 (6.45 %)	0.76 ± 0.60 (1.76 %)	92.98 ± 58.46 (8.64 %)
	72	3.91 ± 3.70 (5.68 %)	0.58 ± 0.46 (1.02 %)	28.92 ± 13.25 (3.38 %)
Glutamine (Gln)	0	0.69 ± 0.05 (1.21 %)	1.88 ± 0.76 (2.87 %)	178.96 ± 16.02 (7.63 %)
	8	0.78 ± 0.40 (1.28 %)	1.74 ± 1.06 (2.94 %)	82.08 ± 44.72 (6.85 %)
	24	0.82 ± 0.16 (1.47 %)	1.54 ± 0.66 (3.44 %)	86.04 ± 38.14 (5.71 %)

(continued on next page)

Table 2 (*continued*)

FAA	Time (h)	Concentrations and percentage		
		Gill (μmol/g)	Muscle (μmol/g)	Hemolymph (μmol/L)
	48	0.49 ± 0.17 (0.80 %)	1.31 ± 0.59 (2.68 %)	48.81 ± 4.12 (4.66 %)
	72	0.87 ± 0.57 (1.50 %)	1.34 ± 0.36 (2.60 %)	81.66 ± 34.79 (9.73 %)

Values represent the mean value with standard deviation in three different individuals. Percentage (%) in parentheses is derived via the ratio of a specific FAA concentration to total 25 FAA concentration in the same sample.

hemolymph of oysters from the control group were 850.28–2363.28 μmol/L through the duration of the experiment (Table 1). And the composition of FAA in hemolymph was slightly different from that in tissues for the relatively higher proportion of Gln. More specially, Tau, Ala, Gly, Glu, Orn, Asp and Gln contributed 72.57–82.70 % of the total content as the predominant FAAs (Table 2), and β-Ala, Lys, and Asn were the majority of the remaining (Table S5).

3.2. Change in FAA contents when exposed to abrupt salinity fluctuations

Generally, the exposure to hypo-salinity and hyper-salinity resulted in decrease and increase of FAA concentrations in tissues respectively. As shown in Table 1, the FAA content in the gill of oysters exposed to 6‰ FSW showed around 50 % decrease at 48 h compared with the control group. When oysters were transferred to 30‰ FSW, it increased by 80 % in total FAA contents since 48 h (Table 1). Interestingly, at 8 h and 24 h, the highest FAA contents of gill samples were detected in oysters exposed to 24‰ FSW, not 30‰ FSW (Table 1). The exposure to 6‰ FSW resulted in a decrease of total FAA contents in adductor muscle since 8 h, but there is no significant difference when exposed to 12‰ FSW (Table 1). As for the exposure to 24‰ FSW, the total FAA in muscle showed a 48 % and 63 % increase at 24 h and 72 h, respectively (Table 1). When transferred to 30‰ FSW, the FAA in muscle increased significantly since 48 h ($P = 0.017$) (Table 1). At the same sampling times, the total FAA concentration in hemolymph of oysters in hypo-salinity or hyper-salinity group was higher than that in the control group, except for that at 24 h and 48 h after the exposure to 6‰ FSW (Table 1). Moreover, the total FAA in the hemolymph of oysters in 12‰ FSW was at a relatively higher level of concentration from 24 h to 48 h (Table 1).

In the gill, the total FAA contents changed mainly due to the change in Tau, Ala, Gly, Asp, Pro and Orn. As shown in Fig. 1A, Tau decreased immediately when oysters were transferred to 6‰ FSW. And the decrease of Tau was 27.27 %– 77.21 % of the total FAA change compared with that in the control group (Table S6). But it was almost constant among gill samples of oysters in 18‰, 24‰ and 30‰ FSW except for that at 48 h after transfer. Interestingly, the Tau concentration of gill samples in 12‰ FSW was close to that in 6‰ FSW at 8 h and 24 h, and then it increased significantly since 48 h, which is similar to Asp (Fig. 1A and B). The trajectory of Gly concentration change in the gills of oysters in 18‰, 24‰ and 30‰ FSW was partially similar to that of Tau, except for the continuous increase 24 h after oysters were transferred to 30‰ FSW. And from 24 h after exposure, the change of Gly in 30‰ FSW group was more than 35 % of the total FAA increase in comparison with that in the control group (Table S6). Moreover, Gly contents decreased by over 60% in gills of oyster in 6‰ and 12‰ FSW when compared with that in the control group (Fig. 1 C). From Fig. 1D, Ala in oyster gills

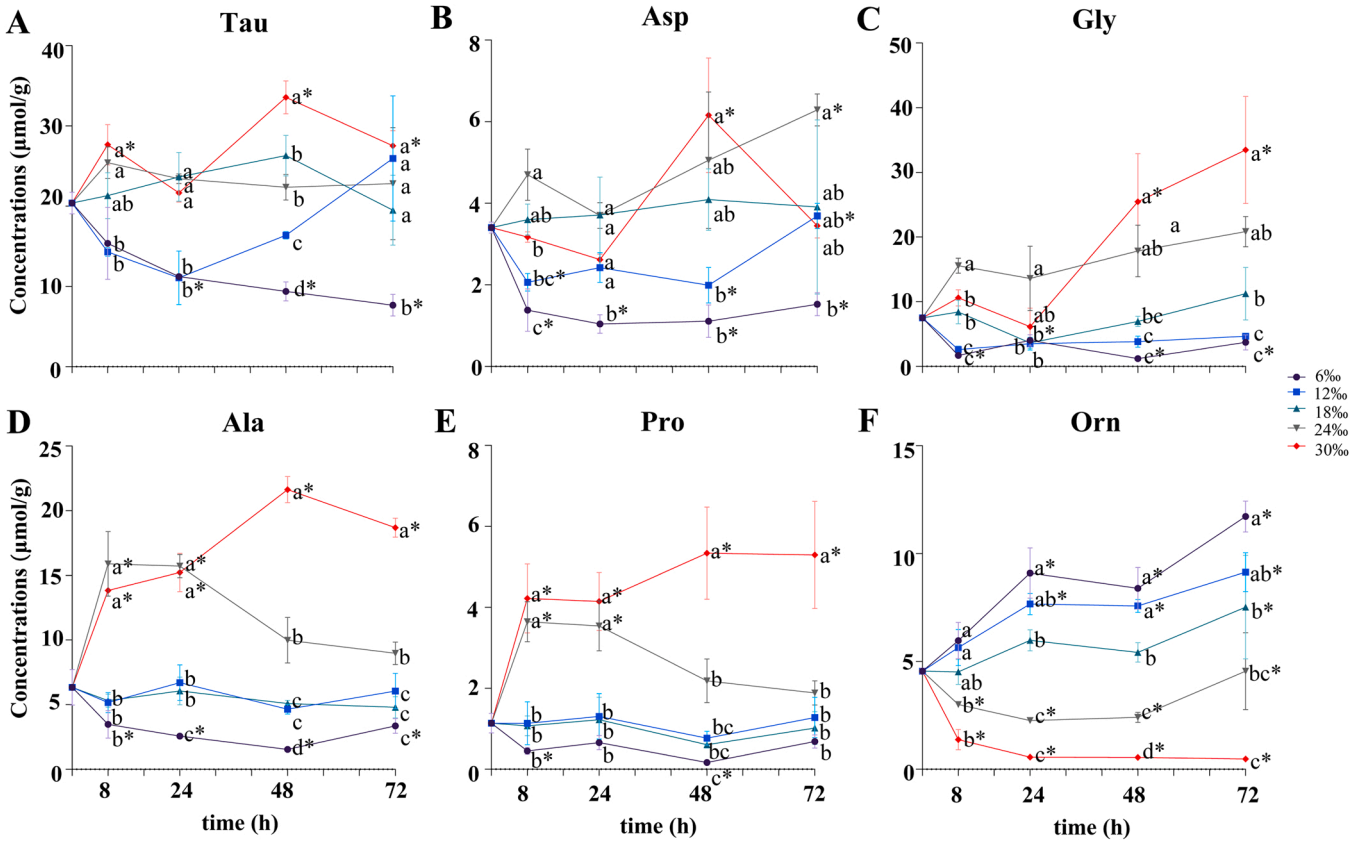

Fig. 1. Change in concentrations of six abundant FAA in the gill of *C. hongkongensis* under different salinities. For illustration, black, blue, green, gray and red represent samples in 6‰, 12‰, 18‰, 24‰ and 30‰ FSW, respectively. Sampling time was indicated at the bottom of corresponding figure. Bars represent standard error. Statistical significance analysis was performed by Duncan's multiple range test and different letters indicate significant differences among FAA contents at same sampling time ($P < 0.05$). Asterisk indicate significant differences between FAA contents at four exposure times and 0 h ($P < 0.05$).

increased immediately after being transferred to 24‰ and 30‰ FSW, and Ala content change was more than of 35% of the total FAA increase at 8 h and 24 h, even nearly 2 folds of the total FAA concentration increase (Table S6). While it decreased by around 36 % at 48 h after exposure to 24‰ FSW. And Ala contents in gills from oysters in 6‰, 12‰ and 18‰ FSW were close, except for that at 24 h and 48 h (Fig. 1D). Similar pattern of concentration change was also detected in Pro, but it was opposite with that of Orn (Fig. 1E and F).

As for adductor muscle, Tau, Ala and Gly also contributed significantly to the fluctuation of total FAA contents under salinity stress. Tau content in muscle decreased by around 40 % from 24 h after the exposure to 6‰ FSW (Fig. 2 A), and the content change was 35.31 % of the total FAA decrease (Table S6). Tau content was almost steady among the muscle samples of oysters in 18‰, 24‰ and 30‰ FSW, but it was higher in samples of oysters in 30‰ FSW than these in 18‰ FSW at 48 h (Fig. 2 A). The decrease under hypo-salinity and consistency under hyper-salinity were also detected in Glu contents of oyster muscle (Fig. 2B). In addition, Ala and Gly concentrations in muscle also varied in the same way with that in gill (Fig. 2C and D), and their co-contribution to the total FAA change under hyper-salinity (24‰ and 30‰ FSW) was more than 60 % (Table S6). But there was a slightly different in trajectories of Pro concentration change between gills and muscles, for the significant variance in Pro contents of muscle samples from oysters in hypo-salinity (6‰ ($P = 0.001$) and 12‰ ($P = 0.01$) FSW) and hyper-salinity (24‰ ($P = 0.01$) and 30‰ ($P = 0.001$) FSW) from 48 h after transfer (Fig. 2E). Like that in oyster gill, high and low level of Orn in adductor muscle were also noticed in hypo-salinity and hyper-salinity, respectively (Fig. 2F).

While Tau and Ala were still the major contributors to the fluctuation of total FAA contents in the hemolymph, Gly, Gln and Orn seemed to contribute equally. From Fig. 3A, Tau increased immediately after the exposure to 6‰ FSW, and it showed no significant difference since 24 h. Ala increased steadily since the exposure to 30‰ FSW, but in 6‰ FSW, it remained at a low level near to that in 18‰ FSW (Fig. 3B). Moreover, Ala contents in hemolymph were at a higher level in 24‰ and 30‰ FSW when compared with that in 6‰ and 18‰ FSW (Fig. 3B). And the contribution of the Ala content change to total FAA change under hyper-salinity ranged from 30.85 % 90.48 % (Table S6). Similar concentration variation could be found in Gly and Pro, although Gly decreased at 48 h after the exposure to 30‰ FSW, and at 72 h after the exposure to 6‰ and 12‰ FSW (Fig. 3C and D). Interestingly, relatively high levels of Gln and Orn were detected in hemolymph samples from oysters in 12‰ FSW, while no significant difference could be found in most situations, except Orn contents in 6‰ ($P = 0.035$) and 30‰ FSW ($P = 0.0001$) at 48 h (Fig. 3E and F).

According to the two-way ANOVA, there was interaction between salinity and treatment time that influenced the content of some FAA in oysters. In the Gly and Ala content variation of oyster gills, a significant interaction was observed between salinity and treatment time ($P < 0.05$) (Table S7). And significant interactions were also observed with regards to Pro, Orn, Valine (Val), Tryptophan (Trp), Ala, Isoleucine (Ile) and Leucine (Leu) in adductor muscle ($P < 0.05$) (Table S7). Additionally, potential interaction between salinity and treatment time was detected in relation to most of the FAA in hemolymph (Table S7).

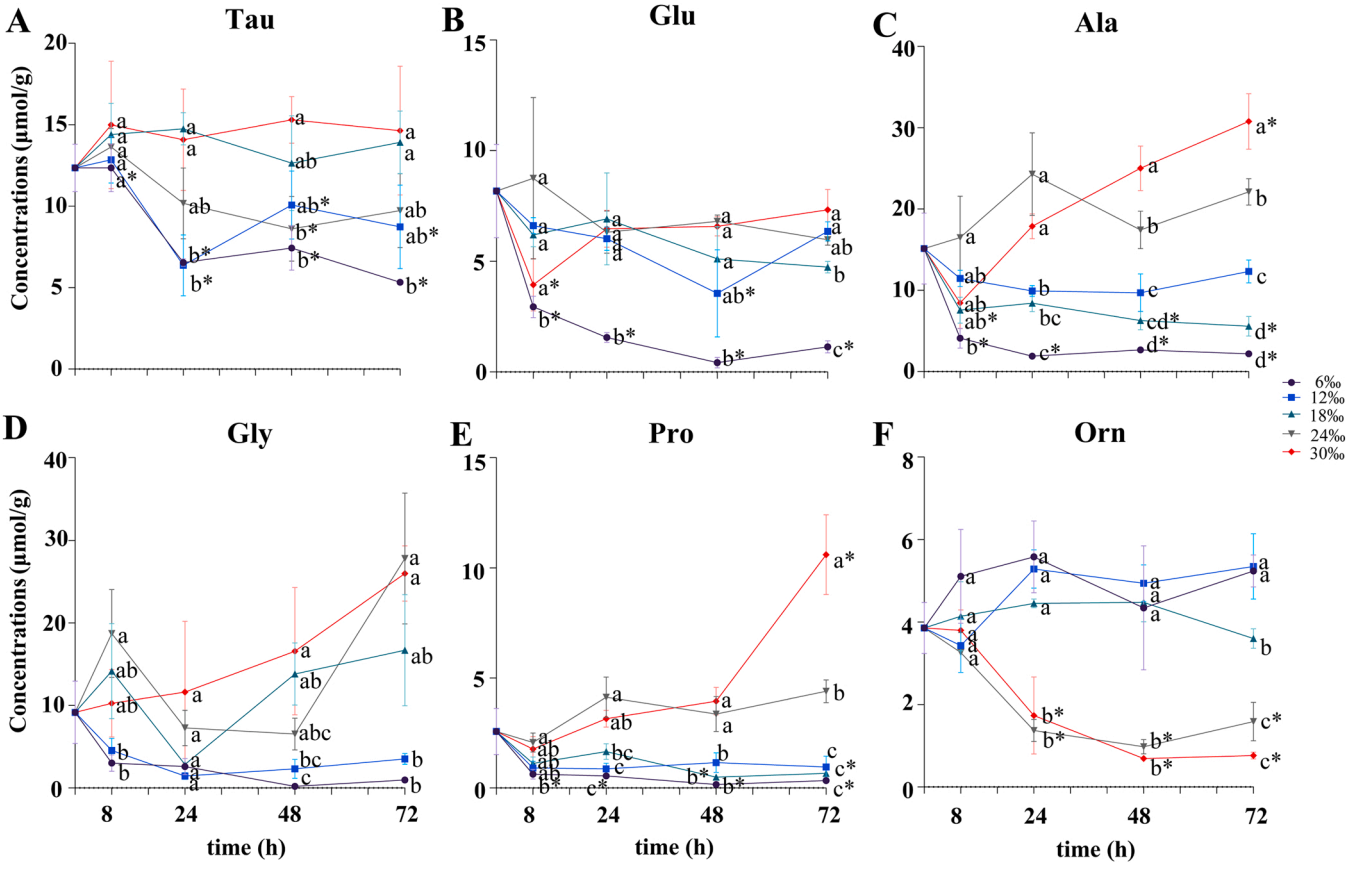

Fig. 2. Change in concentrations of six abundant FAA in the adductor muscle of *C. hongkongensis* under different salinities. For illustration, black, blue, green, gray and red represent samples in 6‰, 12‰, 18‰, 24‰ and 30‰ FSW, respectively. Sampling time was indicated at the bottom of corresponding figure. Bars represent standard error. Statistical significance analysis was performed by Duncan's multiple range test and different letters indicate significant differences among FAA contents at same sampling time ($P < 0.05$). Asterisk indicate significant differences between FAA contents at four exposure times and 0 h ($P < 0.05$).

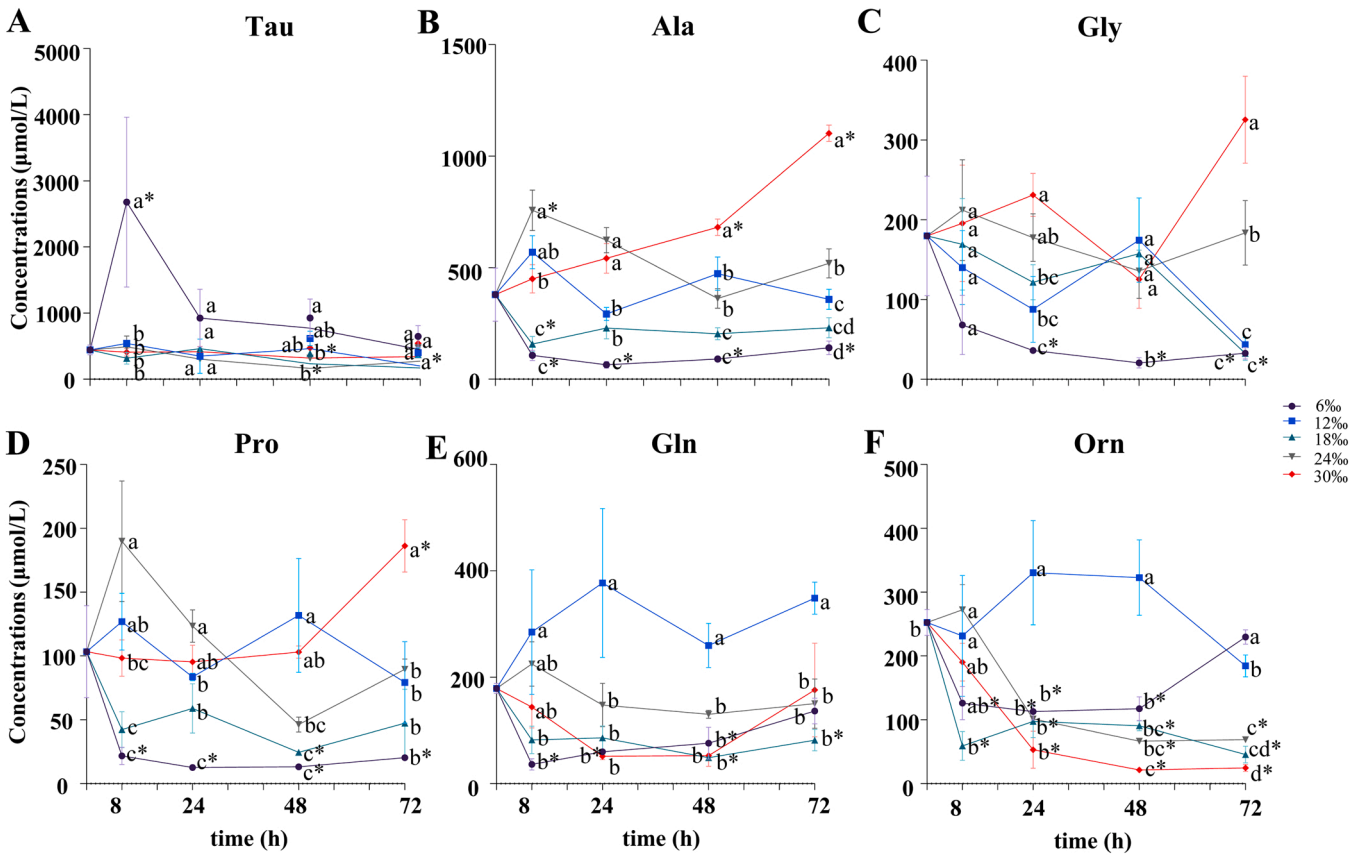

Fig. 3. Change in concentrations of six abundant FAA in the hemolymph of *C. hongkongensis* under different salinities. For illustration, black, blue, green, gray and red represent samples in 6‰, 12‰, 18‰, 24‰ and 30‰ FSW, respectively. Sampling time was indicated at the bottom of corresponding figure. Bars represent standard error. Statistical significance analysis was performed by Duncan's multiple range test and different letters indicate significant differences among FAA contents at same sampling time ($P < 0.05$). Asterisk indicate significant differences between FAA contents at four exposure times and 0 h ($P < 0.05$).

3.3. Identification of major FAA in response to salinity changes based on PCA

The PCA of FAA concentrations in gill generated six PCs with eigenvalues higher than 1, and the first three PCs explained 72.75 % of data variance (Table S8). PC1 retained 49.85 % of data variation and differentiated the gill samples according to the contents of Glu, Gly, Arg, Pro, Histidine (His), Serine (Ser), Tyrosine (Tyr), Lysine (Lys), Methionine (Met), Valine (Val), Phenylalanine (Phe), Tryptophan (Trp), Threonine (Thr), Isoleucine (Ile) and Leucine (Leu) (factor loadings higher than 0.60) (Fig. 4A and Table S8). PC2 explained 16.03 % of the variability in the initial responses and separated the samples based on Tau, Asp, Orn and Ala (Fig. 4B and Table S8). PC3 only explained 6.87 % of the variability, and the factor loadings from PC3 were very low (Table S8). The PCA based on FAA concentrations in muscle generated five PCs with eigenvalues higher than 1, and the first four PCs explained

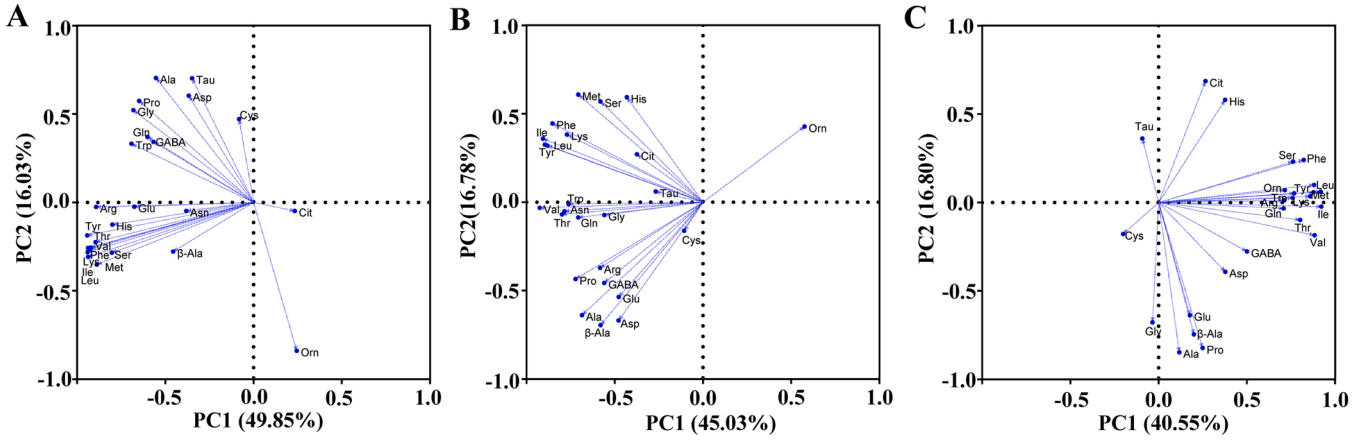

Fig. 4. PCA of samples based on FAA compositions in *C. hongkongensis*. Fig. 4A, B and C showed the first two PC loading vectors generated by PCA based on FAA contents in gill, adductor muscle and hemolymph, respectively. Abbreviation: Tau, Taurine; Asn, Asparagine; Gln, Glutamine; His, Histidine; Ser, Serine; Cit, Citrulline; Asp, Aspartic acid; Glu, Glutamic acid; Gly, Glycine; Arg, Arginine; Cys, Cysteine; Pro, Proline; Tyr, Tyrosine; Orn, Ornithine; Lys, Lysine; Met, Methionine; Val, Valine; Phe, Phenylalanine; Trp, Tryptophan; Thr, Threonine; β-Ala, β-Alanine; Ala, Alanine; GABA, r-Aminobutyric acid; Ile, Isoleucine; Leu Leucine.

75.40 % of data variance (Table S9). PC1 retained 45.03 % of data variation and differentiated the muscle samples according to the contents of Asn, Gln, Pro, Tyr, Lys, Met, Val, Phe, Trp, Thr, Ile and Leu (Fig. 4B and Table S9). PC2 explained 16.78 % of variability and separated the samples based on Asp, Met, Ala and β-Ala (Fig. 4B and Table S9). PC3 and PC4 explained 7.56 % and 6.02 % of variability, respectively, with low factor loadings (except for Tau in PC3). As for hemolymph samples, PCA generated six PCs with eigenvalues higher than 1, and the first four PCs explained 73.05 % of data variance (Table S10). PC1 retained 40.55 % of data variation and differentiated hemolymph samples based on the same FAA as PC1 from PCA of muscle samples (Fig. 4C and Table S10). PC2 explained 16.80 % of variability and separated the samples based on Cit, Glu, Gly, Pro, Ala and β-Ala (Fig. 4C and Table S10). And in PC3 and PC4, there also were similar proportion and factor loadings with the other two PCs generated by PCA based on FAA in muscle samples (Table S10).

4. Discussion

In the present study, 25 FAA in gill, adductor muscle, and hemolymph of *C. hongkongensis* under different salinity stress were quantified by LC-MS/MS, providing a clear and global view of the FAA osmolyte system in this economically important estuarine bivalve. Tau, Ala and Gly were found to be dominant FAA contributing to the total FAA contents that varied with salinities, no matter in gill, muscle, or hemolymph of *C. hongkongensis*, showing high similarity with other species (Hosoi et al., 2003; Sokolowski et al., 2003; Meng et al., 2013; Pourmozaffar et al., 2020). And this may indicate the conservation of the most important FAA that play an important role in the osmotic responses of mollusks. Although the other major FAA, such as Asp, Pro, Gln and Glu, changed significantly in hypo-osmolality and/or hyper-osmolality as found in other mollusks (Hosoi et al., 2003; Meng et al., 2013; Pourmozaffar et al., 2020), their contribution to the total change may be smaller due to their relative low contents. It is interesting that the proportion of Gln in *C. hongkongensis* hemolymph was relatively higher than that in gill and muscle, and the significant content variation with salinity could only be found in the hemolymph. Thanks to a wide variety of FAA analyzed in this study, the abundance of Orn and its contribution to the total FAA variation were detected in gill, muscle and hemolymph of *C. hongkongensis*, which could be found in some mussels but rarely noticed in oysters (Gainey Jr, 1978; Hosoi et al., 2003; Meng et al., 2013; Oliveira et al., 2015). Overall, there is no significant difference in major FAA as osmolyte between gill, adductor muscle and hemolymph for their cooperative relationship in response to osmotic stress, which is discussed in more detail below.

In hypo-osmotic stress response, especially during the early stage, total FAA contents showed a remarkable decrease in gill and muscle but increased in hemolymph of *C. hongkongensis*. It was speculated that the increase of FAA in hemolymph might mainly result from the extrusion of some FAA from gill and muscle to hemolymph, and the FAA decrease in tissues and the excretion of the FAA to the hemolymph likely reduced the osmolarity of the intra-cellular medium to prevent permeation of water into the cell. And FAA contents variation in *C. hongkongensis* exposed to hypo-salinity was primarily due to the change in Tau (27.27–77.21 % of total FAA change in gills when exposed to 6‰ FSW) (Table S6), a well-known osmolyte and generally the most abundant FAA in mollusks (Gilles, 1972; Hosoi et al., 2003; Meng et al., 2013; Nagasaki et al., 2018). After exposure to 6‰ FSW, Tau increased immediately in hemolymph within 8 h and decreased significantly in gill and adductor muscle within 24 h. This result supported the assumption in *C. gigas* that large amount of Tau not used in protein synthesis is accumulated to maintain the osmolality, and to be released in response to hypo-osmolality stress (Hosoi et al., 2003). When *C. hongkongensis* exposed to 12‰ FSW, Ala, Gln, Pro and Orn contributed more in osmotic adaption, as Tau decreased at the early stage and increased from 24 h in tissues but kept consistent in hemolymph all the time. Further studies are required to analyze the difference of FAA contributors between the adaption to 6‰ and 12‰ FSW.

On the other hand, total FAA contents increased in gill, adductor muscle and hemolymph in the response of *C. hongkongensis* to hyper-salinity. This was speculated to increase the intra- and extra-cellular osmolality and subsequently prevent cell shrinking caused by water permeation to the ambient seawater. And in this study, Ala and Gly were mainly responsible for total FAA contents variation in hyper-osmotic adaption (cooperatively contributing more than 55 % of the total FAA change) (Table S6), rather than Tau and Gly or Ala revealed in *C. gigas* (Hosoi et al., 2003; Lee et al., 2004). Tau may not contribute to the hyper-osmotic adaption of *C. hongkongensis*, because it did not change significantly under high-salt stress, which is agreed with a recent report (She et al., 2022). For the immediate increase in gill, Ala was more sensitive than Gly to the hyper-salinity in *C. hongkongensis*. A similar rapid increase of Ala was also detected in the mantle of *C. gigas* exposed to hyper-salinity (Hosoi et al., 2003). Hosoi et al. (2003) assumed that Ala requires less energy for its de novo synthesis than other FAA, and it is advantageous as an osmolyte in mollusks under abrupt hyper-osmotic stress. A significant increase in Pro was also observed during hyper-salinity adaption of *C. hongkongensis* as previous studies on bivalves (Hosoi et al., 2003; Meng et al., 2013; Lin et al., 2016; Pourmozaffar et al., 2020), but its contribution to the total change was smaller due to its lower contents.

It is worth noting that Orn, in this study, showed content variation in the opposite way to most FAA, especially in gill and muscle. As the intermediate of the ornithine-urea cycle (OUC), the concentration variation of Orn in *C. hongkongensis* led to nitrogen excretion and utilization related to FAA metabolism during the osmotic adaption process. Generally, excess amino acids not used in protein production are catabolized to ammonia, which is highly toxic and excreted rapidly or converted to urea or uric acid (Wright, 1995; Ip et al., 2001; Evans et al., 2005). From this, the increase of Orn in the hypo-osmotic adaption of *C. hongkongensis* indicated the OUC pathway might be activated to reduce the ammonia produced by amino acids catabolism, although the functional OUC is still to be investigated in bivalves (Hiong et al., 2004; Leonard et al., 2014; Haider et al., 2020). Whereas the low Orn concentration in hyper-salinity may associate with the active biosynthesis of FAA that requires amino nitrogen. Furthermore, more researches are needed to confirm whether the product of OUC, as well as an osmolyte, urea plays a role in cell volume preservation in *C. hongkongensis* under osmotic stress.

Based on the HPLC-MS/MS, some FAA, such as Tau, Ala and Gly, were identified as major osmolytes for response to hypo/hyper-osmolality, which is necessary for understanding the osmotic regulation mechanism of *C. hongkongensis*. But it remains to be seen whether other FAA (beyond 25 FAA in this study) could participate in the osmotic regulation. In addition, this research provides a useful direction for candidate genes that affect the salinity tolerance of *C. hongkongensis*. And key genes involved in the transportation and metabolism of Tau, Ala and Gly would be investigated furtherly in future studies for the genetic improvement of hyper-salinity tolerance.

5. Conclusion

The objective of this study was to provide a global view of FAA contents variation in *C. hongkongensis* under salinity stress and to identify major FAA contributing mostly as osmolyte. During the adaption to hypoosmotic conditions, total FAA contents showed decrease in gill and muscle, but increase in hemolymph. And Tau contributed most significantly to the hypo-osmotic stress response, especially at the early stage. Total FAA contents increased in all the three kinds of samples from *C. hongkongensis* acclimating to hyperosmotic conditions, with the major contribution of Ala and Gly. It is worth noting that Ala with most rapid increase appears to be an important FAA as an osmolyte for immediate response to hyper-osmolality. Moreover, unusual trend of Orn

concentration was detected, indicating the FAA metabolism during osmotic adaption process. Overall, this result provides basis for the study of intracellular osmoregulation mechanisms in *C. hongkongensis*, which was aimed at directing genetic improvement of the oyster for hypersalinity tolerance.

CRediT authorship contribution statement

XS carried out the sample preparation, the data analysis, and drafted the manuscript. WL and SI participated in the data analysis and manuscript preparation. YD and QL participated in the data analysis. CY conceived of the study, participated in experimental design and coordination, and contributed to the manuscript preparation. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aqrep.2023.101464.

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