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Short Communication

High dietary lipid level promotes low salinity adaptation in the marine euryhaline crab (*Scylla paramamosain*)

Jiaxiang Luo [a], Chen Ren [a], Tingting Zhu [a], Chen Guo [a], Shichao Xie [a], Yingying Zhang [a], Zheng Yang [a], Wenli Zhao [a], Xiangsheng Zhang [a], Jingjing Lu [a], Lefei Jiao [a], Qicun Zhou [a], Douglas R. Tocher [b], Min Jin [a, *]

[a] Laboratory of Fish and Shellfish Nutrition, School of Marine Sciences, Ningbo University, Ningbo 315211, China
[b] Guangdong Provincial Key Laboratory of Marine Biotechnology, Institute of Marine Sciences, Shantou University, Shantou 515063, China

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ABSTRACT

The physiological processes involved in adaptation to osmotic pressure in euryhaline crustaceans are highly energy demanding, but the effects of dietary lipids (fat) on low salinity adaptations have not been well evaluated. In the present study, a total of 120 mud crabs (*Scylla paramamosain*, BW = 17.87 ± 1.49 g) were fed control and high-fat (HF) diets, at both medium salinity (23‰) and low salinity (4‰) for 6 wk, and each treatment had 3 replicates with each replicate containing 10 crabs. The results indicated that a HF diet significantly mitigated the reduction in survival rate, percent weight gain and feed efficiency induced by low salinity ($P < 0.05$). Low salinity lowered lipogenesis and activated lipolysis resulting in lipid depletion in the hepatopancreas of mud crabs ($P < 0.05$). Thus, HF diets enhanced the process of lipolysis to supply more energy. In the gills, low salinity and the HF diet increased the levels of mitochondrial biogenesis markers, the activity of mitochondrial complexes, and the expression levels of genes related to energy metabolism ($P < 0.05$). Consequently, the positive effects of the HF diet on energy metabolism in mud crabs at low salinity promoted osmotic pressure regulation. Specifically, significantly higher haemolymph osmotic pressure and inorganic ion content, as well as higher osmotic pressure regulatory enzyme activity in gills, and gene and protein expression levels of NaK-ATPase were observed in crabs fed the HF diet at low salinity ($P < 0.05$). In summary, high dietary lipid levels improved energy provision to facilitate mitochondrial biogenesis, which increased ATP provision for osmotic pressure regulation of mud crabs. This study also illustrates the importance of dietary lipid nutrition supplementation for low salinity adaptations in mud crabs.

1. Introduction

Aquaculture production of euryhaline crustaceans has recently expanded from the coastal environment to inland regions that have a supply of low salinity ground water (water salinity around 1‰ to 6‰), making use of cleaner water resources and promoting economic development in these areas (Li et al., 2017). Additionally, substantial amounts of land with saline soil have been reclaimed in recent decades as an important increased land resource, thus expanding the economic benefits of these less developed areas (Tan and Kang, 2009; Liu et al., 2020). However, the utilization of saline land is largely confined to saline tolerant plants (Sharma and Minhas, 2005), with lower economic benefits compared to the farming of euryhaline aquaculture species. Therefore, recently, low salinity water has been increasingly used for the aquaculture of euryhaline crustacean species to utilize available water and maximize economic benefits (Li et al., 2017).

Although relatively high economic profit can be obtained from the inland culture of euryhaline crustaceans using low salinity

* Corresponding author.
E-mail address: jinmin@nbu.edu.cn (M. Jin).
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water, there are some problems and potential risks of failure in commercial production, including lower survival rate and growth performance (Chen et al., 2015). The growth and development of aquatic animals in a low salinity environment depends largely on their ability to respond to hypotonic stress and regulate osmotic pressure (Péqueux, 1995; Geng et al., 2016). Recently, the regulatory mechanisms, target organs, physiological and metabolic characteristics of osmotic pressure regulation in euryhaline crustaceans have been studied (Rainbow and Black, 2001; Li et al., 2017). In order to maintain the osmotic pressure of the haemolymph within a stable range, the organs involved in crustacean hypo-osmoregulation include the intestines, antennal glands and, especially, gills (Chung and Lin, 2006a). Extracellular anisosmotic regulation in the posterior gills is one of the crucial osmotic and ionic regulation processes, responsible for the maintenance of osmolality through the actions of ion transporters with epithelial osmotic pressure regulation enzymes like sodium potassium ATP synthase (NaK-ATPase), vacuolar ATP synthase (V-ATPase) and carbonic anhydrase (CA) (Funder et al., 1997; Charmantier, 1998). However, the maintenance of internal ionic homeostasis, normal cellular function and other physiological processes in aquatic animals is energy demanding (Setiarto et al., 2004; Silvia et al., 2004). Importantly, even more energy may be required for osmoregulation at low salinity (Tseng and Hwang, 2008). Some reports indicated that nearly 20% to 50% of metabolic energy was utilized in osmotic pressure regulation in response to hypotonic stress in some aquatic animals (Fielder et al., 2005; Laiz Carrión et al., 2005; Li et al., 2017), leading to lower energy availability for animal growth and development when reared at low salinity (Li et al., 2017).

Thus, the provision of sufficient nutrients/energy through the diet is essential to support and increase the ability of euryhaline crustaceans to regulate osmotic pressure and cope with hypotonic stress. Carbohydrate, lipid and protein are the 3 macronutrients that can supply the metabolic energy necessary for the maintenance of homeostasis, turnover, and tissue and organ growth (NRC, 2011). Carbohydrates can directly fulfill the increased energy demands of the body in some stress situations (Li et al., 2017). Previous studies in marine fish, gilthead sea bream (*gilthead sea bream*) and Senegalese sole (*Solea senegalensis*), indicated that salinity stress enhanced the use of exogenous glucose in gills and other high-energy consuming organs, and promoted gluconeogenesis to satisfy the increased energy requirement (Sangiao Alvarellos et al., 2005; Arjona et al., 2007). Another study in Pacific white shrimp (*Litopenaeus vannamei*) suggested that carbohydrate was a key source of energy in low salinity as high levels of dietary carbohydrate could enhance survival rate and growth performance, at least partly by sparing dietary protein (Wang et al., 2014). Lipids not only play essential roles as membrane components, but also are important and rich sources of energy (Tseng and Hwang, 2008), and studies in Arctic char (*Salvelinus alpinus*) and Atlantic salmon (*Salmo salar* L) showed increased lipid catabolism and decreased lipid accumulation under salinity stress (Nordgarden et al., 2002; Bystriansky et al., 2007). In addition, proteins and their amino acid metabolites can also be key energy sources for aquatic animals acclimating to extreme environments (Tseng and Hwang, 2008). Amino acids are not only oxidized for ATP production, but are also used to synthesize macromolecules in gills and other osmoregulatory organs (Kültz and Jürss, 1993) and, for example, taurine and glycine may play a role in intracellular homeostasis in aquatic animals during salinity challenges (Bystriansky et al., 2007). Li et al. (2017) found that Pacific white shrimp in low salinity had higher growth and condition factors when fed a high-protein diet. Meanwhile, optimal dietary glycine supplementation significantly increased the survival rate of Pacific white shrimp after salinity challenges (Xie et al., 2014).

Mud crabs, *Scylla paramamosain*, are a euryhaline species with good ability to adapt to different levels of salinity (Li et al., 2018), and are one of the new species suitable for development for culture in inland low salinity water (Luo et al., 2021b). Thus, mud crabs are an excellent animal model to study the nutritional and physiological metabolic responses to osmotic stress and the study of adaptation to low salinity in mud crabs could have great practical significance. In preliminary experiments, we fed mud crabs with a control diet, high-carbohydrate diet (HC), high-fat diet (HF) and high-protein diet (HP) (the ingredients and proximate compositions of the experimental diets as shown in Supplementary Table S1) in a medium salinity (23‰) or low salinity (4‰) environment, respectively. The results indicated that only the HF diet could significantly improve resistance to low salinity, and alleviate the reduction of survival rate and weight gain due to hypotonic pressure (the growth performance results as shown in Supplementary Table S2, and the proximate composition in hepatopancreas and muscle as shown in Supplementary Table S3). Therefore, the specific aim of this study was to evaluate the specific effects of high-fat diets on low salinity adaptations, and explore dietary strategies for enhancing osmotic regulation in mud crabs.

2. Materials and methods

2.1. Ethics statement

All experimental procedures complied with the Standard Operation Procedures of the Guide for Use of Experimental Animals of Ningbo University. The study was approved by the Ethics-Scientific Committee for Experiments on Animals of Ningbo University.

2.2. Experimental diets

Two experimental diets, control and HF, were produced, with formulations and proximate compositions shown in Table 1. The control diet was formulated to satisfy the basal nutrient requirements of mud crabs (NRC, 2011) and contained 44.84% protein, 8.22% lipid, 16.09% carbohydrate (nitrogen-free extract) and 16.36 MJ/kg gross energy. The HF diets were formulated to contain

Table 1
Ingredients and proximate compositions of the experimental diets (dry matter, %).

Item	Control	High-fat
Ingredients		
Peruvian fishmeal	38.00	38.00
Soybean protein concentrate	24.00	24.00
Wheat flour	5.70	5.70
Corn starch	10.00	10.00
Cellulose	12.00	7.00
Soybean oil	1.00	6.00
Fish oil	1.00	1.00
Soy lecithin	2.00	2.00
Cholesterol	0.50	0.50
Vitamin premix[1]	0.50	0.50
Mineral premix[1]	1.00	1.00
Choline chloride	0.30	0.30
Monocalcium phosphate	2.00	2.00
Sodium alginate	2.00	2.00
Proximate compositions (analysed)		
Dry matter	85.16	85.74
Crude protein	44.84	44.79
Crude lipid	8.22	13.26
Crude ash	9.84	9.75
Nitrogen free extract	16.09	15.81
Gross energy, MJ/kg	16.36	18.22

[1] Vitamin premix and mineral premix were based on Luo et al. (2021a).

approximately 12% higher energy than control diet, with the additional energy supplied by soybean oil. The experimental diets were produced according to methods reported previously (Luo et al., 2021a). Briefly, all dry ingredients were ground into fine powder with particle size < 177 μm, and micronutrients including minerals and vitamin premixes were added by the progressive enlargement method. Lipid and distilled water (35%, wt/wt) were added to the dry ingredients and the mixtures blended until homogenous in a Hobart-type mixer, and cold-extruded pellets produced (F-26, Machine factory of South China University of Technology) with pellet strands cut into 2 uniform sizes (3 mm and 5 mm diameter) (G-250, Machine factory of South China University of Technology). Pellets were heated at 90 °C for 30 min, air-dried to approximately 15% moisture, sealed in vacuum-packed bags and stored at −20 °C until use.

2.3. Feeding trial and experimental conditions

The feeding trial was carried out in the experimental feed center of Ningbo University (Meishan Campus, Ningbo, China). Juvenile mud crabs were procured from a local farm and, prior to initiation of the feeding trial, cultured in an aquaria (50 L, 48.3 cm × 28.4 cm × 38 cm) in a recirculation aquaculture system (RAS) for 2 wk to acclimate to the laboratory environment. Temperature controlled seawater was continuously purified through a series of filtration treatments including mechanical and bio-filter systems followed by ultraviolet (UV) treatment. Crabs were exposed to an artificial light regime of 12 h light (08:00 to 20:00) and 12 h dark. Low salinity water was obtained by diluting natural seawater with freshwater. Crabs in the low salinity groups were acclimated from 23‰ to 4‰ salinity by decreasing 2‰/d over approximately 10 d prior to the feeding trial. During the entire acclimation and adaptation period, crabs were fed the same diet (approximately 45% protein, 8% lipid). A total of 120 healthy and actively eating crabs were chosen as experimental animals (initial weight 17.87 ± 1.49 g; 60 crabs adapted to medium salinity and 60 crabs adapted to low salinity) were randomly distributed into 120 aquaria with 1 crab in each aquarium to prevent crab aggression as described in detail previously (Luo et al., 2021a, 2021b). Each experimental treatment (MC, medium salinity/control diet; MHF, medium salinity/HF diet; LC, low salinity/control diet; LHF, low salinity/HF diet) was randomly allocated to 3 replicates of 10 crabs each ($n = 30$ crabs per treatment). During the 6 wk experimental period, crabs were hand-fed once daily at 18:00 with a daily ration of 3% to 6% of crab weight, with feeding ration adjusted daily according to actual consumption and residual feed in order to maintain apparent satiation. Faeces and uneaten feed were removed by siphoning daily, and 20% of the aquarium water was renewed each day to maintain water quality. The water quality parameters were measured weekly with mean values as follows (mean ± SEM): salinity (low salinity 4.0 ± 0.25, and medium salinity 23.0 ± 0.70), temperature 26.0 ± 2.90 °C, dissolved oxygen 7.6 ± 0.40 mg/L, ammonia nitrogen 0.04 ± 0.01 mg/L, and pH 7.5 ± 0.30.

2.4. Sample collection

By the end of the experimental feeding period, all crabs had molted at least once and all crabs in each replicate were counted and weighed to determine survival rate, percent weight gain (PWG) and specific growth rate (SGR). Haemolymph samples from 3 crabs in each replicate were taken from the pericardial cavity using a 1-mL syringe, placed into 1.5-mL microfuge tubes (Guangzhou Jet Bio-Filtration Co., Ltd) and centrifuged at 956 × g for 10 min at 4 °C (Eppendorf centrifuge 5810R, Germany). The supernatant was collected and stored at −80 °C until analysis of hematological characteristics. Hepatopancreas and gills samples from the same 3 crabs were dissected quickly and frozen immediately in liquid nitrogen and then stored at −80 °C for analyses of biochemical parameters, protein and gene expression. The same segment of hepatopancreas was sampled in another 2 crabs per replicate and stored in 4% paraformaldehyde solution prior to morphology analysis. Hepatopancreas and muscle from the same 2 crabs were collected in 5-mL centrifuge tubes and stored at −20 °C before the determination of lipid content.

2.5. Proximate composition analysis

Proximate compositions of experimental diets and tissues (hepatopancreas and muscle) of mud crabs were determined following the methods of the Association of Official Analytical Chemists (AOAC, 2006). Moisture content was determined by 105 °C atmospheric pressure drying method. A fully-automatic protein analyzer (FP-528, Leco, USA) was used to measure the protein content according to the Dumas combustion method. Soxtec System HT (Soxtec System HT6, Tecator, Sweden) was used to determine lipid content according to the ether extraction method. For ash content, samples were carbonized fully in an electric furnace, and then incinerated in a muffle furnace at 550 °C for 8 h. Fibre content was measured with an Auto Fibre Analysis System (Fibertec 8,000, FOSS, Germany).

2.6. Hepatopancreas histological determinations

Samples of hepatopancreas were processed and assessed histologically as described by Luo et al. (2021c). Briefly, hepatopancreas samples were dehydrated by treatment with a gradient of ethanol concentrations, embedded in paraffin, sliced into sections of 4 μm, and stained with hematoxylin and eosin (H&E). Images were obtained on a microscope (Olympus, DP72) and the area of hepatopancreatic R cell measured using ImageJ. For each of these indicators, 10 measurements were acquired per tissue sample ($n = 30$ per treatment).

2.7. Lipid class analysis

Hepatopancreas samples were homogenized on ice in 9 volumes (wt/vol) of ice-cold physiological saline, then centrifuged at 1,467 × g for 10 min at 4 °C. Triglyceride (TG), cholesterol (CHO) and non-esterified fatty acid (NEFA) contents of hepatopancreas supernatant and haemolymph of crabs were measured by commercial assay kits according to the manufacturer's instructions (Nanjing Jiancheng Co., Nanjing, China).

2.8. Haemolymph osmolality and inorganic ion detection

Haemolymph osmolality was measured according to the method of Baldes (1934) using a freezing point osmotic pressure analyzer (Fiske 212, AdvancED). The concentrations of inorganic ions, Na^+, K^+ as well as Cl^- in haemolymph were measured according to the methods described by Geng et al. (2016) using related assay kits (Nanjing Jiancheng Co., Ltd). Briefly, Na^+ were assayed its optical density (OD) value at 630 nm by use of the 6-$KSb(OH)_4$ precipitation method. K^+ content was estimated according to the method of NA-TPB reactive precipitation, then estimated spectrophotometrically its OD value in 450 nm wavelength. Cl^- were assayed at 480 nm by use of the $Hg(SCN)_2$ complex methods.

2.9. Enzyme activity and metabolite content assays

Gills sample were homogenized on ice in 9 volumes (wt/vol) of ice-cold physiological saline, and then centrifuged at 1,467 × *g* for 10 min at 4 °C. The supernatant was collected in a fresh sterile tube and used for further analysis of enzyme activity. The activities of NaK-ATPase, CA and V-ATPase in gills supernatant were estimated spectrophotometrically using a crab ELISA kit (Jingkang Bioengineering Co., Ltd, Shanghai, China). The gills supernatant was also used to determine the activities of mitochondrial complexes I, II, III, and V using mitochondrial enzyme activity ELISA assay kits (Meibiao, Jiangsu, China). The ATP levels in the gills were determined using an ATP assay kit (Solarbio, Beijing, China) according to the protocol described previously with some modification (Li et al., 2019).

2.10. MtDNA copy number and gene expression analysis

Gill mitochondrial DNA (mtDNA) copy number was determined by quantitative real-time quantitative PCR (qPCR) using primers for the mitochondrial 16S rRNA gene and nuclear β-actin gene (Supplemental Table S4) as described in detail previously (Han et al., 2021). Total mtDNA of gills was extracted using a tissue DNA isolation kit according to the manufacturer's instructions (Vazyme, Nanjing, China), the related disposable plastic consumables were provided by Jet Biofil (Gaungzhou, China), and real-time qPCR was carried out using a quantitative thermal cycler (Lightcycler 96, Roche, Switzerland). The qPCR assays were performed in a total volume of 20 μL, containing 1.0 μL of each primer, 10 μL of 2 × conc SYBR (Vazyme, Nanjing, China), 2 μL of 1/5 diluted cDNA and 6 μL DEPC-water. The real-time qPCR program was 95 °C for 2 min, followed by 45 cycles of 95 °C for 10 s, 58 °C for 10 s and 72 °C for 20 s.

Expression of mRNA was determined by reverse-transcriptase (RT) qPCR as follows. Briefly, RNA was extracted by TRIzol Reagent and reverse transcribed using HiScript RT SuperMix Reagent kit (Vazyme, Nanjing, China). Specific primers are shown in Supplemental Table S4, and reaction components and cycle conditions of T-qPCR amplification using a Lightcycler 96 (Roche) were as described above. Expression of *β-actin* was found to be unaffected by salinity and dietary treatment and, therefore, was used as a house-keeping gene in the present experiment. Expression levels of mRNA of candidate genes in crabs fed the high energy diets and/or reared at low salinity were reported relative to the expression values in the MC group (crabs fed the control diet at medium salinity). The mRNA expression levels of the candidate genes were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.11. Western blotting analysis

Western blotting of hepatopancreas and gills protein samples was performed essentially according to methods reported previously (Jin et al., 2021). Briefly, total protein was extracted from hepatopancreas and gills using RIPA buffer according to the manufacturer's instructions (Beyotime Biotechnology, China), and protein concentrations determined using a BCA Protein Assay kit according to the manufacturer's instructions (Biosharp, China). Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE) at a constant voltage of 100 V for 1 h. Proteins were then transferred onto a 0.45-μm polyvinylidene difluoride (PVDF) membrane and subsequently blocked with 5% skimmed milk in buffer (Tris buffered saline with Tween, TBST) for 2 h. The membrane was rinsed briefly with TBST prior to probing with primary antibodies: anti-β-ACTIN (ABclonal Technology, China, 1:5,000 dilution), anti-NaK-ATPase (ABclonal Technology, China, 1:1,000 dilution), anti-CA (ABclonal Technology, China, 1:2,000 dilution), anti-LPL (ABclonal Technology, China, 1:500 dilution), anti-CPT1 (ABclonal Technology, China, 1:500 dilution), anti-HSP60 (Abmart, China, 1:500 dilution) at 4 °C overnight. The PVDF membrane was then washed and incubated with secondary antibody (ABclonal Technology, China, 1:5,000 dilution) for 1 h at room temperature. The Western blot images were obtained with a luminescent image analyzer (Tanon 5,200, China) and quantified by Image J.

2.12. Statistical analysis

$$\text{Percent weight gain (PWG, \%)} = 100 \times (W_t - W_i)/W_i$$

$$\text{Specific growth rate (SGR, \%/day)} = 100 \times (\ln W_t - \ln W_i)/t$$

$$\text{Feed efficiency (FE)} = \text{weight gain (g, wet weight)/feed consumed (g, dry weight)}$$

$$\text{Survival rate (\%)} = 100 \times N_t/N_i,$$

where W_t, W_i were the final body weights and initial body weights, respectively; t was the duration of experimental days; N_t and N_i were the final and initial crab numbers, respectively.

Data were expressed as means ± SEM. One-way ANOVA was performed to estimate the significance of differences between different diets within the same salinity level. The significance of differences due to salinity level were determined using an independent samples *t*-test. Two-way ANOVA analysis was applied to calculate the combined effects of salinity and diet on all parameters with the results of two-way ANOVA analysis reported in Supplemental Tables S5–S8. All statistical analyses were carried out using SPSS 19.0.

3. Results

3.1. Effects of salinity and diet on the growth performance of mud crabs

The effects of salinity and dietary nutrients on growth performance, survival and feed utilization are presented in Table 2 and the results of two-way ANOVA are shown in Supplemental Table S5. Compared to medium salinity, low salinity significantly decreased the survival rate of mud crabs fed all diets ($P < 0.05$). In medium salinity, survival rate was not affected by diet ($P > 0.05$), whereas at low salinity, crabs fed the high-fat diet (LHF) exhibited a significantly higher survival rate than crabs fed any of the control diets (LC) ($P < 0.05$). Compared with medium salinity, crabs exhibited significantly lower percent weight gain (PWG) and specific growth rate (SGR), and higher feed efficiency (FE) in low salinity ($P < 0.05$). At medium salinity, PWG, SGR and FE were not significantly affected by diets ($P > 0.05$). At low salinity, crabs fed the HF diet (LHF) had significantly higher PWG, SGR and FE compared to crabs fed the control diet (LC) ($P < 0.05$).

3.2. Effects of salinity and diet on lipid metabolism of mud crabs

The results of two-way ANOVA are shown in Supplemental Table S6, while the specific impacts of the HF diet on lipid metabolism of mud crabs are presented in Fig. 1. Hepatopancreatic R cells are a primary location for lipid deposition and storage in mud crabs, and quantitative histological analysis of the hepatopancreas showed crabs fed the HF diet exhibited higher areas of R cells than those fed the control diet (Fig. 1A and B). Interestingly, when fed the

Table 2
Effects of salinity and diet on survival, growth performance and feed utilization of mud crabs.

Item	Medium salinity		Low salinity	
	Control	High-fat	Control	High-fat
Survival rate, %	89.50 ± 1.90*	87.60 ± 1.90*	66.45 ± 1.78	74.14 ± 2.14#
Percent weight gain, %	99.75 ± 2.56*	103.55 ± 4.67*	80.82 ± 5.51	94.58 ± 1.50#
Specific growth ratio, %/day	1.69 ± 0.01*	1.70 ± 0.05*	1.39 ± 0.06	1.58 ± 0.02#
Feed efficiency	0.48 ± 0.02*	0.51 ± 0.01*	0.32 ± 0.01	0.38 ± 0.02#

* Denotes significantly higher values ($P < 0.05$) for the same diet at different salinity.
# Denotes significantly higher values ($P < 0.05$) between different diets at the same salinity.

HF diet, mud crabs reared at low salinity exhibited lower areas of R cells than crabs reared in medium salinity ($P < 0.05$). The concentrations of lipids in the hepatopancreas, muscle and haemolymph are shown in Fig. 2C–G. Irrespective of salinity, crabs fed the HF diet had significantly higher total lipid content in the hepatopancreas and muscle (Fig. 1C), and higher contents of TG and CHO in haemolymph (Fig. 1D), compared with crabs fed the control diet ($P < 0.05$). In medium salinity, NEFA levels in the hepatopancreas and haemolymph were higher in crabs fed the HF diet compared with crabs fed the control diet ($P < 0.05$; Fig. 1E and F). When fed the control diet, crabs reared in low salinity had significantly lower contents of TG and lipid in the hepatopancreas (Fig. 1G), and NEFA in haemolymph compared to crabs reared in medium salinity ($P < 0.05$). When fed the HF diet, mud crabs reared in low salinity exhibited lower contents of TG, CHO and NEFA in the hepatopancreas, as well as contents of TG and NEFA in haemolymph than crabs reared in medium salinity ($P < 0.05$).

The relative mRNA expression levels of genes related to lipid metabolism in the hepatopancreas are shown in Fig. 1H–J. At medium salinity, the hepatopancreas mRNA expression levels of acetyl-CoA carboxylase 1 (*acc1*), fatty acid synthase (*fas*) and sterol regulatory element binding protein (*srebp*) decreased significantly, whereas expressions of lipoprotein lipase (*lpl*), carnitine palmitoyltransferase 1 (*cpt1*) and fatty acid binding protein 1 (*fabp1*) increased significantly in crabs fed the HF diet (MHF) compared to crabs fed the control diet (MC) ($P < 0.05$). At low salinity, crabs fed the HF diet (LHF) showed significantly lower mRNA expression of *acc1* and significantly higher mRNA expression levels of *lpl* and *cpt1* in the hepatopancreas than crabs fed the control diet (LC) ($P < 0.05$). Crabs fed the C diet had significantly lower mRNA expression levels of *acc1*, *fas* and *srebp* in the hepatopancreas at low salinity than crabs cultured at medium salinity ($P < 0.05$). Irrespective of diet (control or HF), mRNA expression levels of *cpt1* were significantly higher in crabs reared at low salinity compared to crabs reared at medium salinity ($P < 0.05$). Consistent with gene expression, the relative protein concentration of Cpt1 in crabs fed the HF diet was significantly higher than crabs fed the control diet, irrespective of salinity ($P < 0.05$) (Fig. 1L). When fed the control diet, crabs cultured at low salinity (LC) had higher relative protein concentration of Lpl and Cpt1 in the hepatopancreas than crabs cultured at medium salinity (MC) ($P < 0.05$) (Fig. 1L).

3.3. Effects of salinity and diet on energy metabolism of mud crab

The results of two-way ANOVA are shown in Supplemental Table S7, and the specific effects of the HF diet on energy metabolism are presented in Fig. 2. For a given diet, crabs cultured at low salinity (LC and LHF) showed significantly higher relative protein expression of mitochondrial marker protein heat shock protein 60 (HSP60) (Fig. 2A and B) and higher expression of genomic mtDNA copy number (Fig. 2C) than crabs cultured at medium salinity (MC and MHF) ($P < 0.05$). At low salinity, genomic mtDNA copy number was significantly higher in crabs fed HF compared to crabs fed control diet ($P < 0.05$; Fig. 2C). For each salinity, the gills ATP content was significantly higher in crabs fed the HF diet compared to crabs fed the control diet and, for each diet, ATP content was significantly lower in crabs reared at low salinity compared to crabs reared at medium salinity ($P < 0.05$; Fig. 2D). The activities of mitochondrial complex I, complex II and complex V were significantly higher in crabs cultured in low salinity than crabs cultured in medium salinity ($P < 0.05$; Fig. 2E, F & H). At low salinity, crabs fed the HF diet had significantly higher activities of mitochondrial complex I and complex II than those fed the control diet ($P < 0.05$; Fig. 2E and F). The expression levels of genes related to the electron transport chain (cytochrome *b* [*cytb*]) and mitochondrial energy metabolism (sirtuin 1 [*sirt1*], sirtuin *3* (*sirt3*] and nuclear respiratory factor-1 [*nrf1*]) were higher in crabs reared at low salinity compared with crabs reared at medium salinity ($P < 0.05$; Fig. 2I and J). At low salinity, the mRNA expression levels of *cytb* and *sirt1* in the gills were significantly higher in crabs fed the HF diet than crabs fed the control diet ($P < 0.05$; Fig. 2I and J). At medium salinity, crabs fed the HF diet had significantly higher mRNA expression of cytochrome c oxidase II *(cox II)* than those fed the control diet ($P < 0.05$; Fig. 2I).

3.4. Effects of salinity and diet on osmotic regulation of mud crabs

The results of two-way ANOVA are shown in Supplemental Table S8, while the specific effects of the HF diet on osmotic regulation are presented in Fig. 3. Haemolymph osmotic pressure was lower in mud crabs reared in low salinity than crabs reared in medium salinity ($P < 0.05$; Fig. 3A). Diet significantly influenced haemolymph osmotic pressure at low salinity, with crabs fed the HF diet (LHF) showing significantly higher haemolymph osmotic pressure than those fed the control diet (LC) (Fig. 3A), accompanied by significantly higher contents of K^+ and Cl^- in haemolymph ($P < 0.05$; Fig. 3B). In crabs fed the control diet, haemolymph Na^+, K^+ and Cl^- contents in crabs cultured at low salinity (LC) were significantly lower than in crabs cultured at medium salinity (MC) ($P < 0.05$; Fig. 3B).

Significantly higher activities of sodium-potassium-ATPase alpha subunit (NaK-ATPase), carbonic anhydrase (CA) and vacuolar ATP synthase subunit B (V-ATPase), mRNA expression levels of *nak-atpase*, *ca* and *v-atpase* in gills were observed when crabs were reared in low salinity compared to crabs reared in medium salinity ($P < 0.05$; Fig. 3C and D). Accordingly, crabs reared in low salinity displayed higher expression levels of transport protein genes (aquaporin - 1 [*aqp*], sodium hydrogen exchanger [*nah*] and $Na^+/K^+/2Cl^-$ cotransporter [*nkcc*]) than crabs reared in medium salinity ($P < 0.05$). At low salinity, the gills activities of NaK-ATPase and CA, and mRNA expression of *nak-atpase* were significantly higher in the LHF group than in the LC group ($P < 0.05$; Fig. 3C and D).

Of note, the relative proteins levels of NaK-ATPase and CA were up-regulated in crabs at low salinity (LC and LHF) compared to crabs at medium salinity (MC and MHF) ($P < 0.05$; Fig. 3F and G). In

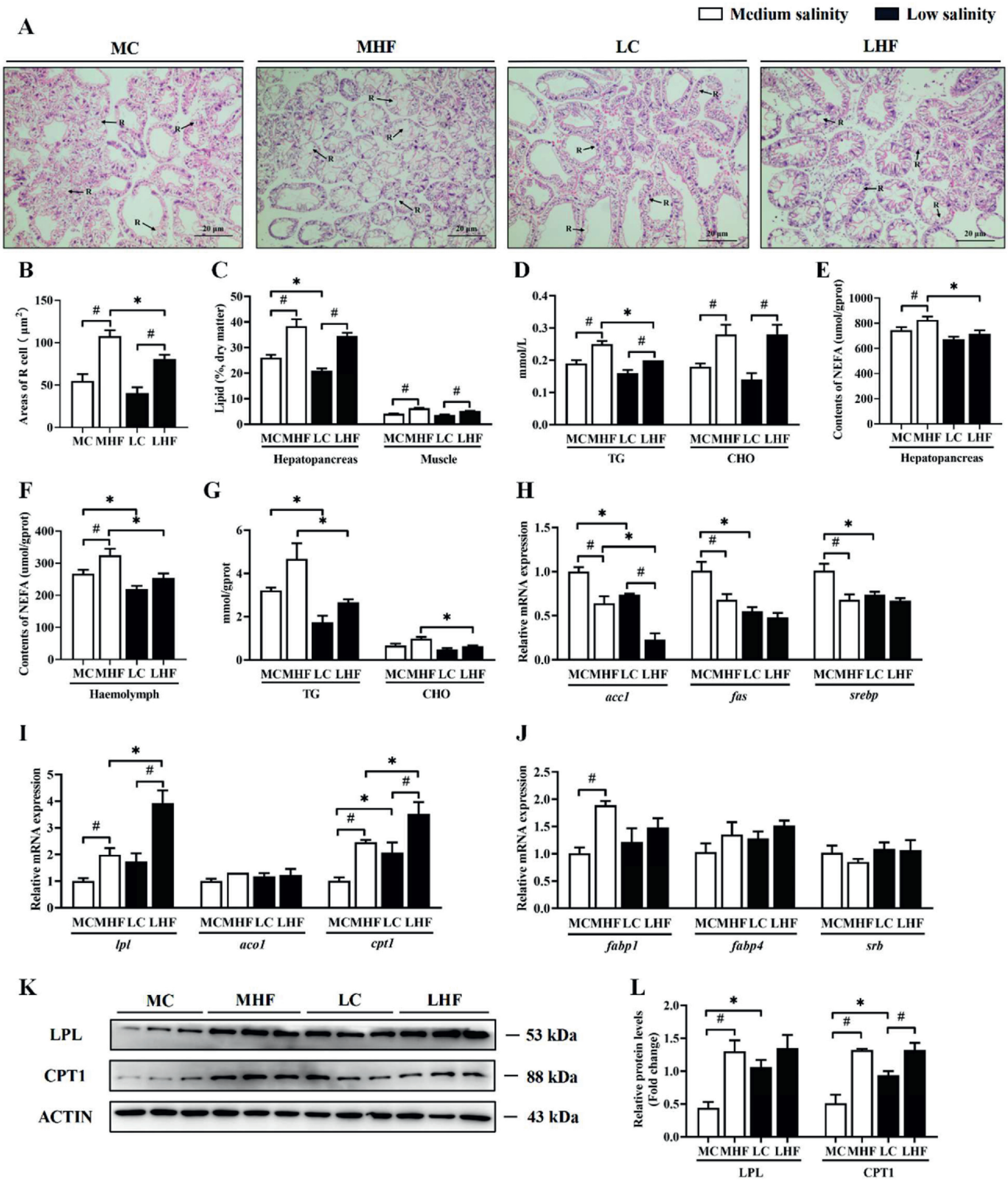

Fig. 1. Effects of salinity and diet on lipid metabolism of mud crabs. (A) Histological sections of hepatopancreas under 100× magnification ($n = 3$). (B) Areas of R (Restzellen) cells in hepatopancreas ($n = 30$). (C) Contents of total lipid in hepatopancreas and muscle. (D) Contents of triglyceride (TG) and cholesterol (CHO) in haemolymph. (E) Content of non-esterified fatty acid (NEFA) in hepatopancreas. (F) Content of NEFA in haemolymph. (G) Contents of TG and CHO in hepatopancreas. (H) Relative mRNA expression levels in hepatopancreas of genes involved in lipid anabolism. (I) Relative mRNA expression levels in hepatopancreas of genes involved in lipid catabolism. (J) Relative mRNA expression levels in hepatopancreas of genes involved in lipid transport. (K and L) Western blotting analysis of relative protein expression levels of LPL and CPT1 in hepatopancreas, and quantitative analysis for relative grey value of Western blotting using ImageJ software. * Denotes significant differences ($P < 0.05$) for the same diet at different salinity. # Denotes significant differences ($P < 0.05$) between different diets at the same salinity.

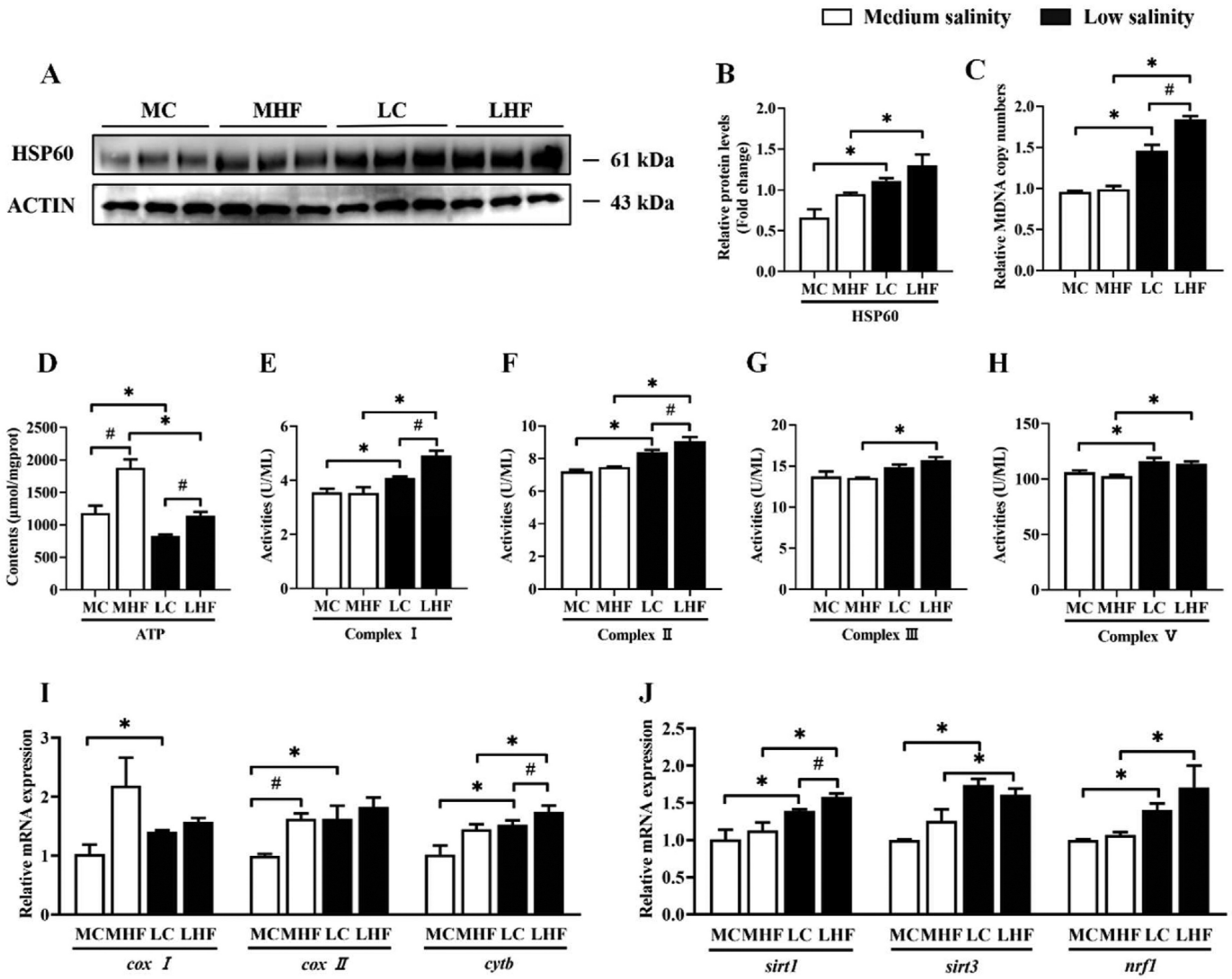

Fig. 2. Effects of salinity and diet on gill energy metabolism of mud crabs. (A and B) Relative protein expression levels of HSP60 in gill, and quantitative analysis for relative gray value of Western blotting. (C) Relative mitochondrial DNA copy number in gill. (D) Content of ATP in gill. (E, F, G, and H) Activities of mitochondrial respiratory chain complex I, II, III and V in gill, respectively. (I) Relative mRNA expression levels of genes related to electron transport chain in gill. (J) Relative mRNA expression levels of genes related to mitochondrial energy metabolism in gill. * Denotes significant differences ($P < 0.05$) for the same diet at different salinity. # Denotes significant differences ($P < 0.05$) between different diets at the same salinity.

addition, at low salinity, crabs fed the HF diet (LHF) had significantly higher relative protein levels of NaK-ATPase compared to crabs fed the control diet (LC) ($P < 0.05$; Fig. 3F and G).

4. Discussion

In recent years, dietary manipulation has become an effective strategy to alleviate salinity stress in aquatic animals (Zhou et al., 2020b). Increased dietary energy level has been reported to exert growth-promoting and protein-sparing effects (Tian et al., 2020). In preliminary experiment, we found that among 3 high-energy diets (high-carbohydrate, high-fat and high-protein), only the HF diet significantly improved low salinity/hypotonic pressure resistance in mud crabs (Supplementary Table S2). Thus, only high dietary lipid (HF diet) was able to improve the growth performance of mud crabs reared under low salinity conditions. In Pacific white shrimp, Zhang et al. (2013) formulated five diets containing 6%, 8%, 10%, 12% or 14% lipid, respectively, and when fed to shrimp at low salinity (6‰ to7‰), results showed that higher dietary lipid levels of 10% to 12% could act as an immune stimulant and therefore benefit the growth and immunity of shrimp in low salinity. Moreover, Chen et al. (2019) also indicated that appropriate supplementation of dietary unsaturated and saturated fatty acids can improve shrimp osmoregulation capacity and thus promote shrimp growth and survival in low salinity (3‰). However, Xu et al. (2018) found that 12% dietary lipid may negatively affect growth and induce oxidative damage in shrimp, but can improve immune defense at low salinity (3‰); 6% dietary lipid cannot support growth and also has no positive impact on immunity for shrimp at low salinity. The variability among crustaceans may be due to differences between the species themselves as, for instance, Pacific white shrimp are omnivorous species, while crabs are carnivorous species (Li et al., 2017, 2018). Different feeding habits may also result in different adaptation patterns to salinity stress. However, the differences reported may also reflect variations in the studies including growth stage, culture conditions and basal diet. Overall, the present results indicated that mud crabs require a higher level of dietary fat (lipid) to adapt to a low salinity environment.

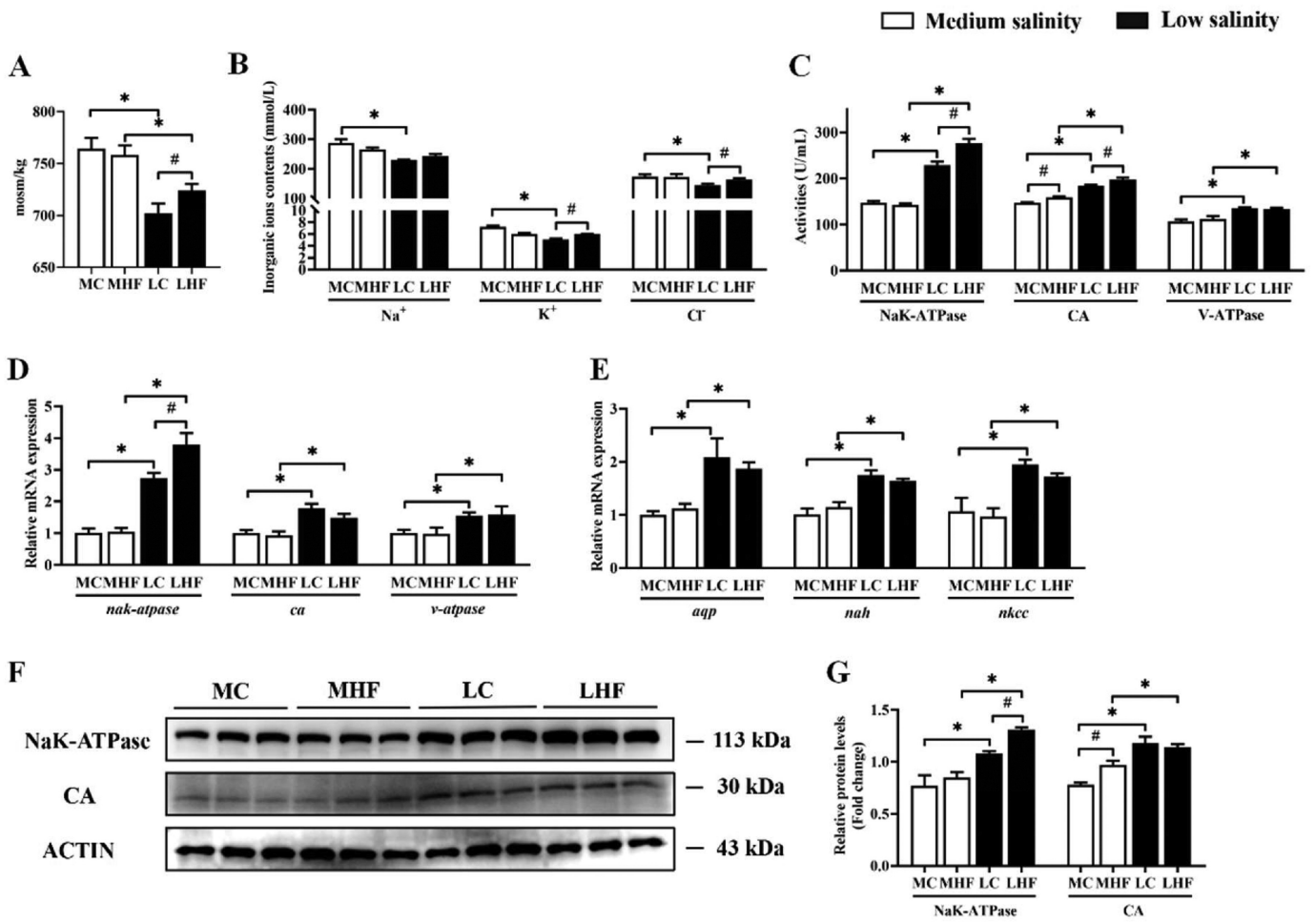

Fig. 3. Effects of salinity and diet on osmotic regulation in gill of mud crabs. (A) Osmotic pressure in haemolymph. (B) Contents of inorganic ions in haemolymph. (C) Activities of osmotic pressure regulating enzymes (NaK-ATPase, CA and V-ATPase) in gill. (D) Relative mRNA expression levels of osmotic pressure regulating enzymes in gill. (E) Relative mRNA expression levels of transport proteins in gill. (F and G) Western blotting analysis of relative protein expression levels of NaK-ATPase and CA in gill, and quantitative analysis for relative grey value of Western blotting using ImageJ software. * Denotes significant differences ($P < 0.05$) for the same diet at different salinity. # Denotes significant differences ($P < 0.05$) between different diets at the same salinity.

The specific mechanisms whereby high lipid intake enhanced the adaptation of mud crabs to low salinity were analyzed further. Dietary lipids play key metabolic roles, particularly in energy metabolism, with lipids providing energy to fuel growth and support turnover and maintenance including homeostatic processes such as osmotic regulation (Luo et al., 2021c). The hepatopancreas plays a central role in lipid metabolism, and is also the primary tissue for lipid deposition in crustaceans (Shi et al., 2020). Histology of the hepatopancreas is a key way to assess the metabolic condition of crustaceans, with the hepatopancreatic R cell being the primary location for lipid deposition and storage (Xiao et al., 2014). In the present study, R cell structures in mud crabs cultured in low salinity showed signs of compressed and decreased vacuolation, with the compression and atrophy of vacuolation in R cells being likely caused by reduced lipid deposition in the hepatopancreas. Consistent with this, biochemical analyses confirmed that the lipid content of the hepatopancreas was significantly reduced by low salinity and demonstrated that lipolysis was enhanced, as evidenced by decreased hepatopancreatic TG. Similar results were reported in Pacific white shrimp, in which low salinity (3‰) decreased hematological TG and high-density lipoprotein content compared to shrimp at high salinity (30‰) (Ke et al., 2014). Li et al. (2007) reported that significantly lower body lipid content was observed in Pacific white shrimp reared at low salinity (3‰) compared to shrimp reared at medium (17‰) or high salinity (32). In contrast, Zhou et al. (2020a) reported that low salinity significantly increased the hematological content of TG and CHO in adult mud crabs after overwintering. Contradictory results have also been reported in fish species. In gilthead sea bream and Arctic char, no statistical differences in hematological (blood) NEFA and TG contents were found among fish at different salinities (Tseng and Hwang, 2008). However, Semra et al. (2013) reported decreased TG contents in plasma of blue tilapia (*Oreochromis aureus*) with the salinity enhanced from 8‰ to 16‰. These inconsistencies in previous studies on lipid metabolism during salinity challenges may be due partially to innate differences in species, but also to variations in acclimation salinities, exposure durations, as well as other experimental conditions. That said, it also illustrates the uncertainty around the role of lipid utilization to fuel osmoregulation upon exposure to environmental osmotic variations. However, for juvenile mud crabs, the results of the present study revealed that low salinity caused lipid depletion in the hepatopancreas, which was confirmed by the expression analysis of genes/proteins related to lipid metabolism. In crabs cultured in low salinity, the mRNA and protein expression levels of Lpl and Cpt1 were down-regulated in the hepatopancreas. While Lpl is rate-limiting for lipolysis and the

removal of lipoprotein TG from the circulation (Mead et al., 2002), Cpt is the rate-limiting enzyme for fatty acid β-oxidation in mitochondria (Sun et al., 2020). Therefore, the results of the present study demonstrated that low salinity enhanced the hydrolysis of TG and β-oxidation of fatty acids in the hepatopancreas of mud crab. In addition, it also enhanced lipid catabolism for the provision of energy as the higher lipid intake of the HF diet further up-regulated Cpt1 expression and enhanced lipid catabolism, promoting fatty acid oxidation and the production of energy for osmotic regulation.

In crustaceans, the gills is the most important tissue for respiration and osmoregulation (Li et al., 2017). Mitochondria are the primary cell organelle for the aerobic generation of energy, with more than 80% of metabolic energy generated in mitochondria (Moyes and Hood, 2003). If energy consumption is increased when dietary energy supply is inadequate, this can be partly compensated and normal energy homeostasis maintained by increased mitochondria number (Bratic and Trifunovic, 2010). In the present study, low salinity and the HF diet both increased gill mitochondrial DNA copy number and mitochondrial marker protein HSP 60 expression level. This may imply that one response to low salinity in mud crabs is increased mitochondria to support the higher energy requirements for osmotic pressure regulation. Mitochondria can produce ATP from fatty acid β-oxidation (Han et al., 2021) in a process on the mitochondrial inner membrane dependent upon the mitochondrial respiratory chain complexes (I-IV), whereby electrons are transferred from electron donors to electron acceptors, passing through the electron transport chain finally to oxygen, and generating ATP (Yuan et al., 2019; Zhang et al., 2020). In the present study, the HF diet significantly increased the content of ATP and activities of Complex I and II of mud crabs. Therefore, the HF diet increased the activity of the mitochondrial respiratory chain complexes leading to enhancing the gill aerobic capacity and, thus, promoting the production of ATP. Cytb is involved in the binding of the quinone substrate and is responsible for transmembrane electron transfer by which redox energy is converted into a proton motive force (Esposti et al., 1993). Sirt1 is the metabolic sensor of energetic status in the cell that regulates cellular physiology and energy demands in response to metabolic inputs, such as fatty acids (Yuan et al., 2019). In low salinity, gill *cytb* and *sirt1* expression levels were both up-regulated significantly in low salinity and when crabs were fed the HF diet, which was consistent with the results obtained for the mitochondrial numbers and respiratory complex activities, and further confirmed that, compared with the control diet, the HF diet improved the mitochondrial activity and energy metabolism of mud crabs in low salinity conditions. While the HF diet also appeared to affect gill energy metabolism of mud crabs when reared in medium salinity, it was not significant. In contrast, Jin et al. (2021) indicated that a high-fat diet resulted in lower hepatic Sirt1 expression in teleost black seabream (*Acanthopagrus schlegelii*) and, in mammals, a high-fat diet led to both a decrease in the mitochondrial quinone pool and modification of mitochondrial lipid composition (Vial et al., 2011). Despite species differences, the present study has implied that energy metabolism and strategies were differentiated in mud crabs in different salinity conditions, and that crabs utilize lipid more efficiently in low salinity. In effect, mud crabs had a higher dietary lipid requirement at low salinity than at medium salinity.

Osmotic pressure regulation is a process that controls cellular permeability and concentrations of ions (Arjona et al., 2007). Among them, Na^+, K^+ and Cl^- are the most important contributors to haemolymph osmotic pressure, accounting for 76% to 94% of the total osmotic pressure (Castille and Lawrence, 1981). Regulation of ion permeability in crustaceans mainly depends on ion transporter proteins on epithelial membranes of the gills, among which NaK-ATPase is particularly important and plays an important role in regulating salinity and ion balance (Huang et al., 2019). NaK-ATPase uses the energy generated by hydrolyzing ATP to power the exchange of 3 intracellular Na^+ with 2 extracellular K^+ and can also affect the activities of other enzymes in the gill epithelium (Takeo et al., 2015). In addition, several studies have demonstrated that carbonic anhydrase (CA), vacuolar ATP synthase subunit B (V-ATPase), aquaporin (AQP) and Na—K—Cl cotransporter (NKCC) play important roles in regulating osmotic pressure and maintaining ion balance in crustaceans (Chung and Lin, 2006; Xu et al., 2017; Huang et al., 2019). In the present study, crabs reared at low salinity showed an increased osmotic regulation response in the gills, with enhanced expression levels of NaK-ATPase, CA, V-ATPase, AQP and NKCC compared to crabs in medium salinity. These results were somewhat in accordance with other studies in euryhaline crustaceans, such as Chinese mitten crabs (*Eriocheir sinensis*) (Long et al., 2018), Pacific white shrimp (Li et al., 2017) and oriental river prawn (*Macrobrachium nipponense*) (Huang et al., 2019). Furthermore, it was noteworthy that mud crabs fed the HF diet showed significantly increased enzyme activity, as well as mRNA and protein expression levels of NaK-ATPase when reared at low salinity. As mentioned above, the osmotic regulation processes mediated by NaK-ATPase (and other ATPases) are consequently highly energy demanding and so the HF diet may also contribute to activated expression of NaK-ATPase simply by the provision of a higher dietary energy supply compared to crabs fed the control diet. Overall, the present study has provided some insight to the compensatory osmotic regulation mechanism in mud crabs reared at low salinity, and demonstrated a positive correlation between lipid nutrition and NaK-ATPase during osmotic regulation.

5. Conclusion

Low salinity culture of mud crabs is an effective way to both increase aquaculture production and the utilization of saline-alkali land. The present study indicated that mud crabs had higher dietary lipid requirements in a low salinity environment and that high dietary lipid levels could effectively mitigate the reduced survival rate and lower weight gain induced by low salinity. Further analysis showed that high dietary lipid levels promoted lipid catabolism in low salinity, and maintained energy supply through activating mitochondrial biosynthesis and energy metabolism. Importantly, high dietary lipid levels could activate NaK-ATPase and enhance mitochondrial energy metabolism to promote osmotic regulation. A deeper knowledge of the relationship between lipid nutrition and osmotic regulation will provide further novel insights into the adaptation of euryhaline species, like mud crabs, to low salinity.

Author contributions

Jiaxiang Luo, **Min Jin** and **Qicun Zhou:** conceived and designed the experiments. **Jiaxiang Luo**, **Chen Ren**, **Jingjing Lu** and **Tingting Zhu:** performed the experiments. **Jiaxiang Luo:** analyzed the data. **Jiaxiang Luo**, **Chen Guo**, **Zheng Yang** and **Chen Ren:** contributed reagents/materials/analysis tools. **Jiaxiang Luo**, **Xiangsheng Zhang**, **Tingting Zhu** and **Lefei Jiao:** prepared the crab diets. **Jiaxiang Luo**, **Tingting Zhu**, **Yingying Zhang** and **Wenli Zhao:** collected the samples. **Jiaxiang Luo**, **Min Jin**, **Douglas R. Tocher**, and **Qicun Zhou:** wrote the paper. All authors contributed to and approved the manuscript.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately

influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Appendix supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2022.10.004.

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