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Variation of specific proteins, mitochondria and fatty acid composition in gill of *Scylla serrata* (Crustacea, Decapoda) under low temperature adaptation

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Abstract

The mud crab *Scylla serrata* is an important commercial crustacean inhabiting estuarine water along the coast of southeast China. Metabolism in the gill is affected continuously by fluctuating water temperature and, therefore, the ability to cope with temperature change is essential to maintain physiological function. This experiment was conducted to help understand the mechanism of low temperature adaptation in *S. serrata* gill. In this study, 40 healthy juvenile male *S. serrata* from the same broodstock were grouped randomly into four groups, which were kept at 5 °C, 10 °C, 15 °C and 27 °C, with the same feeding regime during a 3-week adaptation period. Two-dimensional electrophoresis of the proteome was conducted to separate the specific proteins responsible for low temperature adaptation. Variations in the mitochondria were observed using transmission electron microscopy, and fatty acid composition was determined using gas chromatography. The results showed that different numbers of specific proteins were expressed under different low temperature adaptation, with more expressed at 5 °C and 10 °C than at 15 °C. Mitochondrial morphology also varied under different low temperature adaptation, but there was no linear relationship between microbial density and adaptation temperature. The composition of different fatty acids in the gill varied considerably with adaptation temperature, but elongation of the carbon chain and transition from fatty acids occurred at lower temperatures. Thus, changes in the specific proteins, mitochondria and fatty acid composition of the gill were the positive effects of low temperature on metabolism, leading to improved adaptation ability in *S. serrata.*

Keywords: Fatty acid; Gill; Low temperature adaptation; Mitochondria; Proteomic; *Scylla serrata*

1. Introduction

The mud crab *Scylla serrata* is an important eurythermal species inhabiting temperate coastal waters, estuaries and tidal areas which are exposed to frequent and abrupt changes in environmental temperature. As temperature changes, the biochemical, physiological, morphological and/or behavioural modulations enable the crab to adapt to the ambient temperature. The mechanism of temperature adaptation involves regulation of specific enzymes or specific proteins (defined by Kimmel and Bradley, 2001). Bradley et al. (1988) suggested that *Eurytemora affinis* was adapting at the protein level to changing salinity and temperature. The first protein response to be observed was the increase of hsp70 during

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salinity stress (Bradley and Ward, 1989). Gonzalez and Bradley (1994a) theorized that specific proteins related to salinity stress might be present in copepods. Gonzalez and Bradley (1994b) were able to demonstrate differential protein expression in response to salinity and temperature. Kimmel and Bradley (2001) separated the specific proteins expressed during treatment under various salinity and temperature regimes and suggested that least stress would involve the fewest protein changes.

Two-dimensional gel electrophoresis (2D-E) was first described by Kenrick and Margolis (1970), combining the primary separation of proteins by isoelectric point with a subsequent separation by molecular weight. Separation of proteins in two dimensions and digital-imaging analysis, which is known at present as proteomics, has allowed us to further examine protein expression in *S. serrata*. These two independent properties of proteins make it possible to separate complex mixtures of proteins from different organisms or tissues (Klose, 1975; O'Farrell, 1975). The result of 2D-E is a gel containing a "map" of protein spots visualized by staining techniques. The protein maps can be used to distinguish different biological states of an organism or tissue. The maps are also useful in isolating key proteins that can be identified later for function. Recently, analysis of the proteome has been applied to environmental and ecological questions (Bradley et al., 1994, 1996; Kültz and Somero, 1996; Shepard and Bradley, 2000; Shepard et al., 2000).Our goal was to determine protein expression signatures for *S. serrata* exposed to various temperatures in order to determine how temperature affected adaptation in *S. serrata*. Gels from different treatments were matched to determine the key proteins present or absent. An overall change in protein expression due to salinity stress has been seen in another crustacean, the Chinese crab *Eriocheir sinensis* (Gilles, 1977).

As temperature changed, specific proteins responded to the different temperatures (Lilley et al., 2001), not only in terms of protein content but also in terms of protein species. In order to understand the mechanism of cryo-adaptation in an organism, it is necessary to identify the specific proteins expressed at different temperatures. In this study, we examined changes in protein expression in *S. serrata* exposed to extremely low temperatures. Compared to the normal temperature, the number of proteins present or absent, or the protein changes observed, was specific to the particular low temperature treatment.

Critical temperature threshold (Tc) is an important characteristic for various marine invertebrate species and fish, which has been defined as being characterized by the transition to an anaerobic mode of metabolism, once temperature reaches low or high extremes (Zielinski and Pörtner, 1996; Sommer et al., 1997; van Dijk et al., 1999); and this is detailed in the reviews of Pörtner et al. (1998, 1999). The occurrence of a Tc value shift has been correlated with adaptation to latitude and to seasonal temperature, and was viewed as the threshold characteristic for the geographical distribution of a species. Comparison between temperate and subpolar species shows that a shift in both low and high Tc is associated with a change in mitochondrial density, which falls as the temperature rises and increases as the ambient temperature falls (Sommer and Pörtner, 1999).

Organisms inhabiting seas in the Antarctic region, where the marine fauna is constantly exposed to low temperatures between −1.9 °C and −1 °C, must have developed special physiological adaptations to overcome the adverse effects of cold temperatures on metabolism. In Antarctic fish a compensatory increase in whole animal aerobic capacity is reflected in higher mitochondrial densities (Dunn, 1988). Compared to temperate species, the drop in the low Tc to below polar temperatures on evolutionary time scales is also linked to a drop in the upper Tc and a narrowing of the tolerated temperature window (Pörtner et al., 1998). In conclusion, mitochondrial performance is a characteristic element in adaptation to temperature.

In order to address the effect of low temperature adaptation on mitochondrial functions in *S. serrata*, an experiment was designed to study mitochondrial density in the gill of a eurythermal crab, *S. serrata*, which is widely distributed along the southeast coast of China. Low temperature effect on mitochondria was studied in order to determine when low temperatures possibly disrupted mitochondrial function.

Fatty acids, including combined fatty acids in neutral lipid and phospholipids and free fatty acids, play an important role in performing physiological function. The effects of temperature acclimation have been studied in many species, including marine (Chapelle, 1978; Farkas and Nevenzel, 1981) and freshwater (Cossins, 1976; Farkas, 1979) crustaceans. The status of fatty acid composition in crustaceans may reflect health status (Li et al., 1994) and gonad development (Cheng et al., 2001), as well as the physiological regulation to the variation of environment factors (Cossins, 1976; Chapelle, 1978; Farkas, 1979; Farkas and Nevenzel, 1981; Pruitt, 1990). Recent studies, especially, have indicated that fatty acid composition in the mud crab, *S. serrata*, is related to low temperature (Kong et al., 2005, 2006, 2007).

The mud crab *S. serrata* is an important commercial crustacean, which is distributed along the coast of southeast China. At present, *S. serrata* is cultivated ubiquitously in this region because of the decrease in shrimp culture due to disease outbreaks. Some farmers have tried

to introduce *S. serrata* to northern China, but it is difficult for them to live at low temperature. Therefore, *S. serrata* must be acclimated to low temperature. In order to help understand the mechanism of physiological and biochemical adaptation to low temperature, this experiment to study the different protein response, mitochondria density and fatty acid composition under low temperature adaptation was carried out, and may serve as a guide for the future culture of *S. serrata*.

2. Materials and methods

2.1. The experimental animals

Juvenile *S. serrata* from one brood (one mother) were sampled from a farm in Zhaoan, Fujian province, where they had been fed and managed under the same conditions. After they were brought back to the laboratory, 60 healthy male *S. serrata* without damage were selected, after adaptation, 40 *S. serrata* were placed randomly into four groups of 10 (Table 1).

2.2. Temperature adaptation

For each group, the test was conducted in ten replicate 2 L plastics tanks (one *S. serrata* per tank; 1.5 L of water per tank) based on the design described by Cheng et al. (2005), and the groups of tanks were placed in four light-regulated (a cycle of 12-h light/dark) containers . The test *S. serrata* was cultured in filtered and aerated seawater with a salinity of 25 PSU.

After one week incubation, the temperatures were decreased at a rate of 1 °C/12 h to the experimental temperatures, which were 5±1.5 °C, 10±1.5 °C and 15±1.5 °C. Room temperature (27±2.5 °C) was used as the control. Once reaching the experimental temperature, the adaptation experiment was started. *S. serrata* were fed using a formulated commercial crab diet and short-necked clam (*Ruditapes philippinarum*). The seawater was changed (maintaining the correct temperatures) every three days.

Table 1
The size of the juvenile *S. serrata* in the experiment

	Adaptation temperature			27 °C
	5 °C	10 °C	15 °C	
Carapace width (cm)	3.06±0.27	2.88±0.24	2.95±0.24	2.86±0.18
Carapace length (cm)	2.16±0.21	2.09±0.18	2.13±0.18	2.08±0.17
Body weight (g)	5.04±1.18	4.60±1.13	4.69±1.07	4.53±0.49

2.3. Two-dimension electrophoresis (2-DE)

After three weeks, *S. serrata* were taken and their gills rapidly isolated for analysis. The gills were rinsed in 0.9% sodium chloride and completely cleaned, and then, 0.2 g prepared gills were homogenized in 1 mL 10% ice-cold trichloroacetic acid (TCA) solution (dissolved in acetone, containing 0.1% DTT) with a glass-teflon Potter homogenizer. To be convenient for protein denaturalization, the homogenate was stored at −20 °C overnight, and centrifuged by an Eppendorf 5810R (made in Germany) at 20,000 *g* (RCF) at 4 °C for 50 min. After the supernatant was discarded, 1 mL acetone (containing 0.1% DTT) was added to suspend the pellet, which was treated ultrasonically for 2 min to mix completely, and then centrifuged at 20,000 *g* at 4 °C for 50 min. The same operation was conducted again to wash the pellet, but without ultrasonic treatment. The pellet was dried in a cool-drier, and then 0.8 mL dissolution buffer added (7.5 mol/L urea, 2 mol/L thiourea, 4% CHAPS, 40 mmol/L Tris base, 40 mmol/L DTT, 2% IPG buffer (pH3-10)). The pellet was dissolved and mixed completely at 4 °C for 2 h, the mixture was centrifuged at 20,000 *g* at 4 °C for 50 min, and then the supernatant stored at −80 °C for analysis. Protein concentration was measured based on colorimetric methods described by Bradford (1976).

Immobiline linear IPG strips (pH3∼10) (from Amersham Pharmacia Biotech Inc) were used for isoelectrophoresis focusing using IPGphor (Amersham Pharmacia Biotech Inc.). 250 μL rehydration solution (8 mol/L urea, 2% CHAPS, 0.5% IPG buffer, 0.002% bromoblue, a little DTT added just before use) containing 150 μg proteins was used for each 13 cm strip. After the protective membrane of the strip was removed, it was placed correctly and immersed completely in the rehydration solution. Then, 2 mL mineral oil was added to cover the surface of the rehydration solution. After low voltage rehydration, IEF was auto-performed.

Temperature and maximal electric current during IEF were controlled at 20 °C and 0.05 mA per IPG strip, respectively. After IEF was finished, strips were taken out and balanced in solution I (50 mmol/L Tris–HCl (pH8.8), 6 mol/L urea,30% glycerol, 2% SDS, 0.01 g / mL DTT) for 15 min, with DTT added just before use, then in balance solution II (50 mmol/L Tris–HCl (pH 8.8), 6 mol/L urea,30% glycerol, 2% SDS, 0.04 g /mL IAA) for 15 min, with IAA added just before use.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed with gel (160 mm×180 mm×1.5 mm, T=10%, C=2.6%) in a SE600 (Amersham Pharmacia Biotech Inc.). Constant

electric current was adopted according to the protocol (Step1, 10 mA per gel for 15 min; Step2, 20 mA per gel for 5.5 h). AA/BA stored solution (T=30.8%, C=2.6%), separation gel buffer solution (1.5 mol/L Tris–HCl, pH 8.8) and electrophoresis buffer solution (25 mmol/L Tris, 192 mmol/L Glycine, 0.1% SDS, pH 8.3) were prepared according to the manual (Amersham Pharmacia Biotech Inc.). A protein marker (Fermentas com.), used in SDS-PAGE, was added in a single lateral slot. Proteins were separated and identified by silver staining method (Rabilloud, 1999). Gel maps were scanned and recorded by Labscan3.01 software in an Image Scanner (UMAX Power1100). 4 replicate gels for the same sample were obtained under same condition. Of these, 3 better gels were used to analyze using image analysis software Imagemaster 2D Elite according to the manual respectively. Protein spots matching between different gels was performed using image analysis software.

Expression quantity of each protein recognized by image analysis software was determined using the amount corresponding to each spot as a percent of total spots recognized on the gel after normalization performed by Imagemaster 2D Elite software. For the same spot, the average value of 3 replicate was used as the quantity of

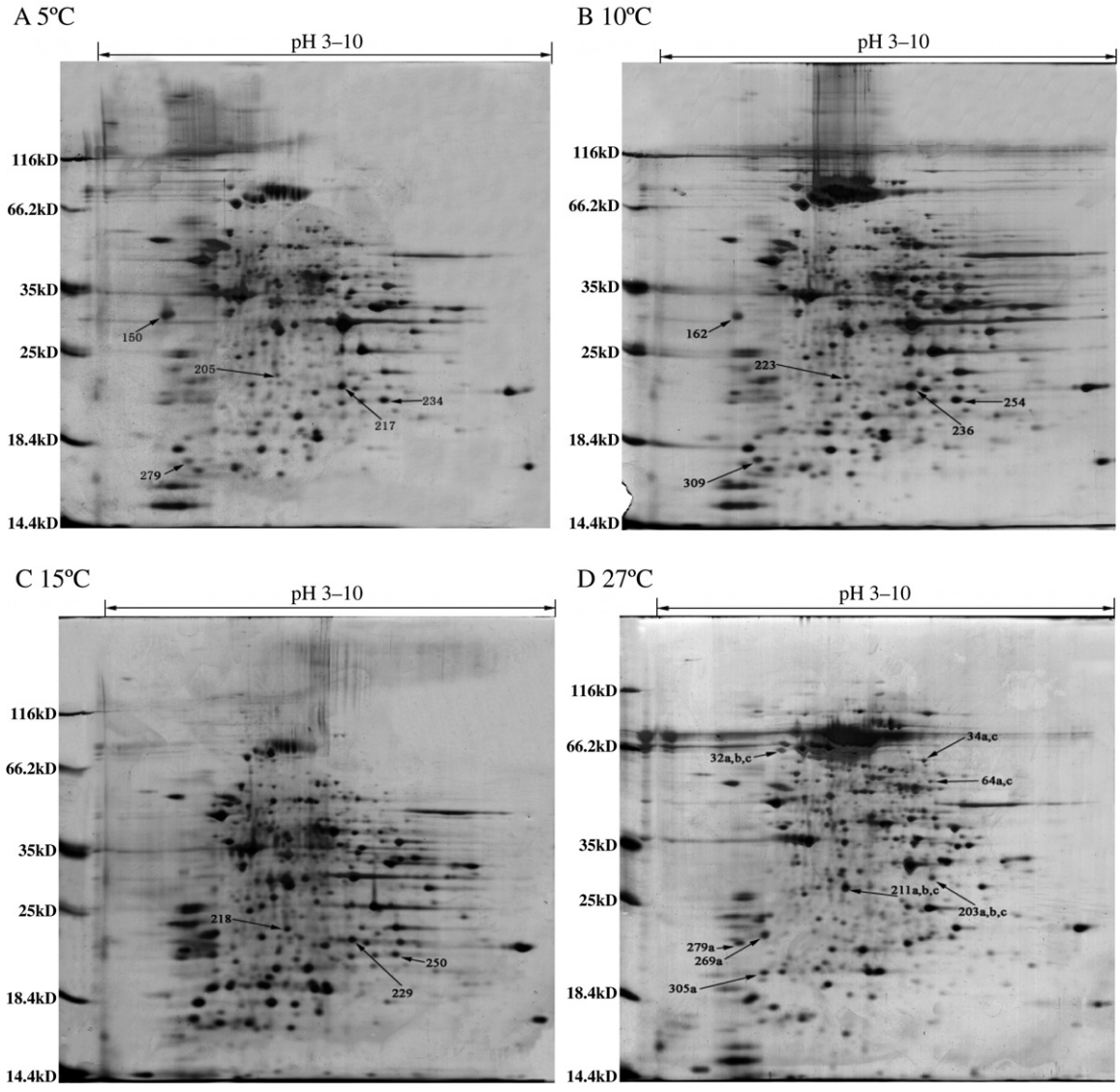

Fig. 1. 2D-E map of *S. serrata* gill under low temperature adaptation. Note: Numbers at the left are protein markers, IPG strip (3→10) is showed on the upper side. Compared with the control group, specific proteins are marked by arrowheads. No. in arrowhead terminal represents the code for specific proteins produced by ImageMaster 2D Elite image analysis software. In D, specific proteins only appeared at 27 °C; a, b and c sign the present specific proteins compared with 5 °C, 10 °C and 15 °C, respectively.

Table 2
Changes of specific proteins in *S. serrata* gill under 5 °C adaptation

5 °C				27 °C			
Prot. no.	Content	p*I*	MW	Prot. no.	Content	p*I*	MW
150	0.62	4.00	30.84	32 (a, b, c)	0.31	4.92	65.53
205	0.22	5.77	22.91	34 (a, c)	0.24	7.08	59.72
217	0.88	6.78	21.93	64 (a, c)	0.14	7.19	50.30
234	0.85	7.45	20.87	203 (a, b, c)	0.40	7.21	29.11
279	0.38	4.37	17.53	211 (a, b, c)	0.82	5.89	27.45
				269 (a)	0.63	4.67	21.58
				279 (a)	0.58	4.28	21.01
				305 (a)	0.45	4.66	19.45

Note: Prot. no. represents the codes for various protein spots on the gel, which was produced by ImageMaster 2D Elite analysis software; Specific proteins at 5 °C only present at 5 °C, and absent at 27 °C; Specific proteins at 27 °C only appeared at 27 °C (a, b and c represent comparison with the 5 °C, 10 °C and 15 °C group, respectively). Protein content is a relative quantity (the percent of single protein spot volume to total volume of all protein spots recognized by image analysis software after normalization). p*I* represents isoelectric point; MW represents molecular weight. This note also applies to Tables 3 and 4.

individual protein expression. To be convenient for analysis, the quantity of protein expression was designated high expression (protein content $\geqq 0.50\%$), medium expression ($0.50\% >$ protein content $\geqq 0.10\%$), low expression ($0.10\% >$ protein content $\geqq 0.05\%$) and trace expression (protein content $< 0.05\%$).

2.4. Observation of mitochondria

S. serrata was sacrificed and the fifth pair of gills picked out. About 0.5 cm was cut from the middle of the gill, and rinsed in 2.5% glutaraldehyde (AMERESCO Com.). It was fixed for 2 h, and then 2 mm^3 of tissue was rinsed in 0.01 mol/L PBS. Before being observed, the gill was fixed in osmic acid for 2 h, and then, dehydrated in a series of alcohol, embedded in EPSON815, dissected and observed under electron transmission microscopy. The number of mitochondria in one visual scope under 1×10^4 magnifying microscope was thought of as one unit, and counted for ten visual scopes.

Table 3
Changes of specific proteins in *S. serrata* gill under 10 °C adaptation

10 °C				27 °C			
Prot. no.	Content	p*I*	MW	Prot. no.	Content	p*I*	MW
162	0.60	4.14	30.57	32 (a, b, c)	0.31	4.92	65.53
223	0.21	5.80	22.61	203 (a, b, c)	0.40	7.21	29.11
236	0.95	6.77	21.77	211 (a, b, c)	0.82	5.89	27.45
254	0.85	7.47	20.80				
309	0.52	4.46	17.62				

2.5. Determination of fatty acids

Fatty acid determination was based on conventional gas chromatography. The fatty acid standards used in this study were C14:0 (Myristic acid); C16:1ω7 (Palmitoleic acid); C 16:0 (Palmitic acid); C18:2ω6 (Linoleic acid); C18:1ω9 (Oleic acid); C18:3ω3 (Linolenic acid); C18:0 (Stearic acid); C19:0 (nonadecanoic acid); C20:4ω6 (Arachidonic acid, ArA); C20:5ω3 (Eicosapentaenoic acid, EPA); C20:1ω9 (Eicosenoic acid); C20:3ω3 (Eicosatrienoic acid); C20:0 (Arachidic acid); C22:6ω3 (Docosahexaenoic acid, DHA); C22:1ω9 (Erucic acid); C22:0 (Behenic acid); C24:1ω9 (Nervonic acid); C24:0 (Lignoceric acid) and C26:0 (Cerotic acid) (from Fluka corp., American); C19:0 was used as the inner standard. The hexane and methanol were chromatographically pure.

After *S. serrata* was dissected, gills were removed and rinsed in 0.9% sodium chloride. The gills were dried in an oven at 60 °C for 16–20 h and then completely ground. The gill powder was sealed in a tube and stored at −20 °C for analysis. Gas chromatography (SP-3420 model, made in China) with an FID detector and an SE-54 (30m × 0.22 mm) elasticity quartz capillary column was used to determine the content of the various fatty acids. The detailed protocol was described by Kong (2004).

2.6. Statistical analysis

Statistical analysis was performed using Microsoft Excel 2003. The significant difference between the adaptation groups and the control was analyzed by One-way analysis of variance (one-way ANOVA) and student's *t* test. The significance level was $p = 0.05$ or $p = 0.01$.

3. Results

3.1. The specific proteins in S. serrata gills under different adaptation temperatures

The specific proteins expressed in *S. serrata* gills varied at different adaptation temperatures (Fig. 1).

Table 4
Changes of specific proteins in *S. serrata* gill under 15 °C adaptation

15 °C				27 °C			
Prot. no.	Content	p*I*	MW	Prot. no.	Content	p*I*	MW
218	0.39	5.84	23.37	32 (a, b, c)	0.31	4.92	65.53
229	0.66	6.85	22.41	34 (a, c)	0.24	7.08	59.72
250	0.57	7.52	21.25	64 (a, c)	0.14	7.19	50.30
				203 (a, b, c)	0.40	7.21	29.11
				211 (a, b, c)	0.82	5.89	27.45

Table 5
Match of specific proteins in *S. serrata* gill under different temperature adaptation

Adaptation temperature			27 °C
5 °C	10 °C	15 °C	
150 M	162 M		32 a, b, c
205 M	223 M	218 M	34 a, c
217 M	236 M	229 M	64 a, c
234 M	254 M	250 M	203 a, b, c
279 M	309 M		211 a,b,c
			269 a
			279 a
			305 a

Note: Numbers in the table are the codes of the specific proteins. Proteins marked by M in the same line are the matching proteins (Protein matching was performed by image analysis software). a, b and c represent comparisons with the 5 °C, 10 °C and 15 °C groups, respectively.

There were 13 different proteins between *S. serrata* at 5 °C and 27 °C (Table 2). Five of them were expressed in *S. serrata* at 5 °C but not 27 °C, and the other eight proteins which were expressed at 27 °C did not appear at 5 °C. The specific proteins were mostly of high or medium content. Based on p*I*, they were either neutral or acid proteins and none of specific proteins was alkaline protein. In terms of MW, specific proteins at 5 °C were almost all in the range 17.53–30.84 kD; while those at 27 °C were in the range 19.45–65.53 kD.

There were eight different proteins between the 10 °C and 27 °C group (Table 3). Five specific proteins expressed at 10 °C were absent at 27 °C, while three proteins were only expressed at 27 °C. Protein content was within 0.21–0.95; based on p*I*, they were neutral or acid proteins, around 4.14–7.41; and their MW values were almost all in the range 17.62–65.53.

There were eight different proteins between *S. serrata* at 15 °C and 27 °C (Table 4). Three specific proteins were expressed only at 15 °C; the five other proteins were expressed at 27 °C and did not appear at 15 °C. The content of these proteins was in the range 0.14–0.82; based on p*I*, they were around 4.92–7.52; and their MW values were in the range 21.25–65.53.

Compared with *S. serrata* at 27 °C, most of specific proteins expressed at different low temperature were consistent (Table 5). Specific proteins expressed at 5 °C and 10 °C matched completely (it referred to the same proteins), and three of them exactly matched specific proteins expressed at 15 °C.

3.2. Mitochondrial change in gill lobe cells under low temperature adaptation

Morphology, distribution and density of mitochondria varied in the gill lobe cells of *S. serrata* under low temperature adaptation (Fig. 2). Morphologically, they were

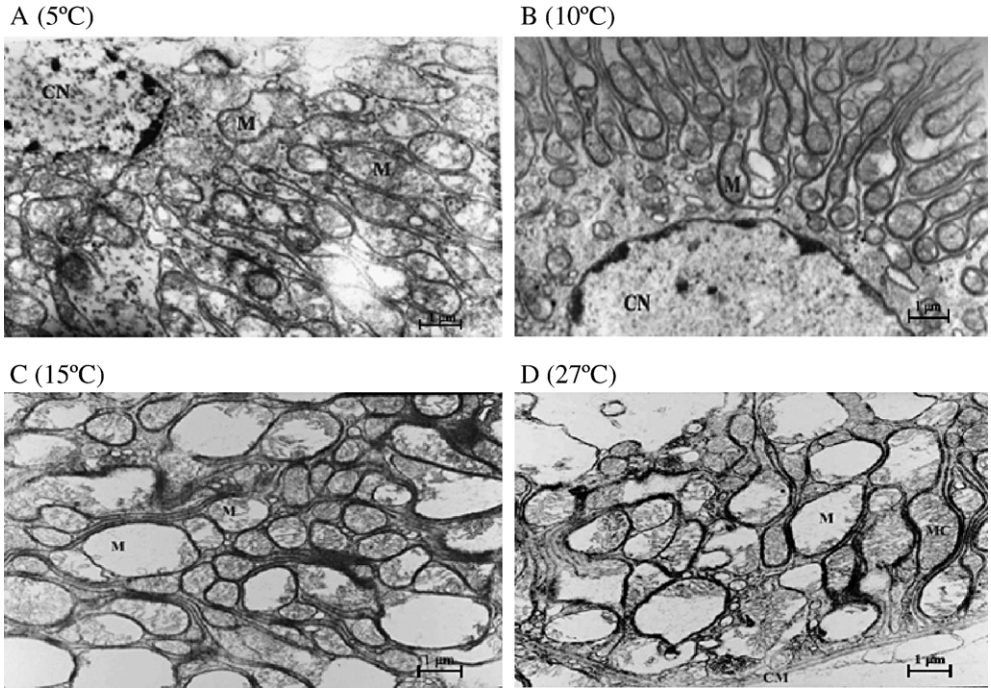

Fig. 2. Mitochondria in *S. serrata* gill cells under different temperature adaptation. Note: Magnification is 1×10^4. The scale is represented by a 1 μm transverse bar. M: mitochondria; MC: Mitochondria cristae; CN: Cell nucleus; CM: Cell membrane.

Table 6
The mitochondria density in the *S. serrata* gill under different temperature adaptation

Temperature	5 °C	10 °C	15 °C	27 °C
Mitochondrial density	65±20	83±29*	64±19	56±13

Note: Statistics of mitochondria density are based on the number of mitochondria per visual scope; all values are given as mean ± standard variation; "*" means that there was a significant difference between the adaptation group and the 27 °C control group ($p<0.05$).

round, ovate or irregular in shape. Inner folding of the mitochondrial membrane, which forms many saclike structures (so-called finger heave, FH), was observed in gill lobe cells. In these sacs, many mitochondria formed mitochondrial clusters. The majority of mitochondria at 5 °C and 10 °C were round or ovate. Mitochondria at 15 °C and 27 °C were relatively larger than those at 5 °C and 10 °C. Mitochondrial density at low temperature was higher than at 27 °C(Table 6), but there was only a significant difference between *S. serrata* at 10 °C and 27 °C ($p<0.05$).

3.3. The composition of fatty acids in S. serrata gills under low temperature adaptation

Fatty acid composition in *S. serrata* gills is shown in Table 7. Compared with the 27 °C group, the content of

Table 7
Changes of the fatty acid composition in *S. serrata* gill under low temperature adaptation (relative proportion, %) (means±standard deviation) ($n=3$)

Fatty acid	Adaptation temperature			27 °C
	5 °C	10 °C	15 °C	
C14:0	3.06±0.87*	2.95±0.88*	2.61±0.70*	1.08±0.28
C16:1ω7	4.66±0.95	4.24±0.83	6.05±0.14	5.23±1.04
C16:0	9.25±1.05**	17.12±1.86	23.62±2.56	22.71±2.41
C18:2ω6	4.72±1.47	2.66±0.65	3.32±0.70	2.18±0.76
C18:1ω9	23.68±2.55	19.70±2.07*	23.22±1.73	27.43±2.36
C18:3ω3	3.26±0.85	4.77±0.87	3.68±1.32	3.76±0.97
C18:0	19.48±2.44**	15.91±1.80*	13.36±2.48	10.37±2.00
C20:4ω6	3.08±0.80	4.03±0.82	3.47±0.56	2.17±0.83
C20:5ω3	4.87±0.85	6.86±0.77	6.64±1.35	5.73±1.09
C20:1ω9	2.39±0.67	2.77±0.85	1.45±0.65*	3.84±0.76
C20:3ω3	2.04±0.90	1.11±0.37	0.24±0.21	0.55±0.32
C20:0	2.17±0.80	2.08±0.89	0.00±0.00	3.64±0.61
C22:6ω3	9.52±1.19	9.46±1.47	7.93±1.88	8.25±1.56
C22:1ω3	3.80±0.73*	2.78±0.70	1.87±0.67	1.52±0.49
C22:0	2.31±0.62	2.07±0.78	1.24±0.68	1.09±0.64
C24:1	–	–	–	–
C24:0	–	–	–	–
C26:0	1.70±0.63	1.50±0.67	1.30±0.54	0.43±0.29
ΣC14	3.06±0.87*	2.95±0.88*	2.61±0.70*	1.08±0.28
ΣC16	13.91±1.39**	21.36±1.67*	29.67±2.70	27.94±1.53
ΣC18	51.15±4.04*	43.03±3.34	43.57±3.08	43.75±0.73
ΣC20	14.55±1.57	16.84±1.52	11.80±1.44	15.93±1.31
ΣC22	15.63±0.32*	14.31±0.21*	11.04±2.96	10.86±1.94
ΣC24	–	–	–	–
ΣC26	1.70±0.63*	1.50±0.67	1.30±0.54	0.43±0.29
ΣSFA	37.97±1.13	41.63±1.27	42.14±2.73	39.31±2.63
ΣMUFA	34.52±1.37	29.49±1.17**	32.58±1.26*	38.04±3.64
ΣPUFA-ω6	7.80±0.83	6.69±0.32	6.79±0.16	4.35±0.19
ΣPUFA-ω3	19.70±0.33	22.20±1.12*	18.49±3.64	18.30±1.59
ΣUFA	62.03±1.13	58.38±1.27	57.86±2.73	60.68±2.63
ΣSFA/ΣUFA	0.61±0.03	0.71±0.04	0.73±0.08	0.65±0.07
ω6/ω3	0.40±0.05**	0.30±0.02*	0.38±0.07*	0.24±0.02
EPA+DHA	14.40±1.92	16.32±2.17	14.57±3.20	13.99±2.60
EPA/DHA	0.51±0.06*	0.73±0.05	0.84±0.06*	0.70±0.06

Note: In Cn_1:n_2ωn_3, n_1 represents C atom number, :n_2 represents olefinic bond number, ω represents olefinic bond position, n_3 represents the first C position of the olefinic bond counted from methyl C; ΣCn represents the sum of the fatty acids which have n C atoms; ΣSFA represents the sum of the saturated fatty acids; ΣMUFA represents the sum of the single unsaturated fatty acids; ΣPUFA represents the sum of the poly unsaturated fatty acids; ΣUFA represents the sum of the unsaturated fatty acids; "–" represents no determination; " tr" represents the trace fatty acids (<0.1%); "*" represents significant difference ($p<0.05$) compared to the 27 °C group; "**" represents highly significant difference ($p<0.01$).

C14:0 increased significantly under low temperature adaptation ($p<0.05$); the content of C16:0 decreased significantly at 5 °C ($p<0.01$); the content of C18:0 increased gradually with the fall of temperature, while statistical significance was shown only at 5 °C and 10 °C ($p<0.01$, or $p<0.05$); there was no significant difference in C20:4, C20:5 and C22:6 between the adaptation groups and the 27 °C group ($p>0.05$).

In contrast to the 27 °C group, the content of ΣC16 decreased significantly at 5 °C and 10 °C ($p<0.01$ or $p<0.05$); the content of ΣC18 increased significantly at 5 °C($p<0.05$); the content of ΣC20 did not show a significant difference ($p>0.05$); and the content of ΣC22 increased gradually with the decrease of temperature, but showed significant difference only at 5 °C and 10 °C ($p<0.05$).

Compared with the 27 °C group, the content of ΣSFA,ΣUFA and the ratio of ΣSFA/ΣUFA did not show any significant difference ($p>0.05$); the content of ΣMUFA decreased significantly at 10 °C and 15 °C; the content of ΣPUFA-ω3 increased at low temperature, but only showed significant difference at 10 °C; the ratio of ω6/ω3 increased significantly at low temperature ($p<0.01$ or $p<0.05$); the content of EPA+DHA did not show any significant difference ($p>0.05$); and the ratio of EPA/DHA increased significantly at 15 °C ($p<0.05$), but decreased significantly at 5 °C ($p<0.05$).

4. Discussion

4.1. Specific protein expression under low temperature adaptation

As environmental temperature changed, the biochemical reaction and physiological metabolism of organisms will respond to these changes in order to improve their ability to cope with environmental changes. Although mRNA differences may reflect the expression of proteins in responding to environmental changes to some degree, it does not reflect the protein panorama completely (Gabor and Maleszka, 2001). Separation (using 2-D electrophoresis in the proteome protocol) of specific proteins, which appear under specific environmental conditions, is very important to understand the functioning of proteins in the process of adaptation (Kong et al., 2005).

In this study, the results showed that the five specific proteins expressed at 5 °C were the same as those expressed at 10 °C, and three of them also matched the three specific proteins expressed at 15 °C. Obviously, more specific proteins occurred at the lower temperature adaptation. In general, most of those specific proteins were metabolic enzymes, stress proteins and signal regulation proteins, which regulated the metabolism of adaptation to the environment. Gills play an important role in respiration and osmotic pressure regulation. When temperature decreased, specific proteins expressed at low temperature would improve the adaptation ability of *S. serrata* to the specific environment.

Ninety percent of the cell protein content is due to 10% of protein species (Gabor and Maleszka, 2001; Zuo et al., 2001). Although protein separation based on 2D-E is currently considered as an effective method (Gygi et al., 2000; Görg et al., 2000), because of limitations in this method, it is difficult to separate the trace proteins. In this experiment, certain trace proteins must have been lost in the process of separation based on pH (3–10) strips. Only the obviously different proteins between the adaptation and the control group could be determined as specific proteins in this study, and so the further research on specific proteins is necessary, based on the narrow pH scope and the long electrophoresis time.

4.2. Changes of mitochondria in the gill under low temperature adaptation

Mitochondria are important organelles in eukaryotes, and play an important role in energy metabolism. Most (90%) of the ATP produced in oxide phosphorylation is to supply energy for various aspects of physiological metabolism. For poikilothermic animals, the critical temperature threshold (Tc) is related to environmental temperature, and could show a downshift under low temperature adaptation (Pörtner et al., 1998, 1999). At the same time, it has been proved that Tc was correlated with mitochondrial density (Sommer and Pörtner, 1999).

Compared with the control group (27 °C), mitochondria increased significantly only at 10 °C ($p<0.05$). Energy metabolism in the gill could be compensated for by other pathways during temperature decrease. Thus, with decrease of temperature, mitochondrial increase at 10 °C became the major way to compensate energy metabolism at low temperature. Interestingly, mitochondria at 5 °C did not show any significant difference. This was probably due to mitochondrial apoptosis. The reason for this is as follows: the major energy in an organism is produced in the respiratory chain in mitochondria, but in the process of energy production, many reactive oxygen species (ROS) may be produced and accumulated, and the ability to remove ROS decreased at low temperature, so the mitochondria would be damaged and apoptosis induced by the superfluous ROS.

In fishes inhabiting Arctic waters, the compensation ability for aerial metabolism (Dunn, 1988) and oxygen enzyme activity (1.5–5 fold that of temperate fishes) (Crockett and Sidell, 1990) are higher than those in other waters in order to improve adaptation ability to changes in environmental temperature. At the same time, it has been proved that mitochondrial density in the cell was much higher. In *S. serrata* living in subtropical estuarine waters, mitochondria increased under low temperature adaptation, but it was confined to a certain temperature. These results implied that, only at a certain low temperature, *S. serrata*, living in subtropical waters, could be acclimated.

4.3. Changes of fatty acid composition

Fatty acid composition in the gill varied under low temperature adaptation. The content of long chain fatty acids increased at low temperature, and this result was in keeping with the suggestion by Pazos et al. (2003). The content of saturated fatty acids decreased significantly at low temperature, and this result agreed with the research by Cuculescu et al. (1995) in *Cancer pagurus* and *Carcinus maenas*.

The content of C16:0 in the gill decreased gradually with the fall of temperature, and this was due to dehydration to supply energy or transition to form long chain fatty acids. Compared with the 27 °C group, the content of ΣC16 decreased significantly at 5 °C and 10 °C ($p<0.01$ or $p<0.05$); whereas the content of ΣC22 increased significantly at 5 °C and 10 °C ($p<0.05$). In general, increase of long chain fatty acids is implemented by the elongation of short chain fatty acids, and this was why the transition occurred among the different fatty acids. These results agreed with the conclusion reported in fish by Zhu and Cao (2002). At 5 °C and 10 °C, *S. serrata* did not intake any food, so no exogenous fatty acids were supplied, and thus, changes of fatty acids were likely to be the reaction of dehydration and transition among the fatty acids.

Some fatty acids perform important physiological functions, such as C20:4, C20:5 and C22:6, and so there was no significant difference between the adaptation group and the control group ($p>0.05$); ΣSFA, ΣUFA and ΣSFA/ΣUFA also showed no significant difference ($p>0.05$). These results showed that the stability of these fatty acids was very important to perform physiological functions. ΣPUFA-ω3 is the major fatty acid (Pazos et al., 2003), which plays a critical role in physiological metabolism at low temperature, however, in this study, only at 10 °C did ΣPUFA-ω3 increase significantly ($p<0.05$), and this is a puzzle to be solved in the future.

4.4. Positive effects of low temperature adaptation

The gill of *S. serrata*, which is exposed directly to the environment, plays an important role in respiration and osmotic regulation. As water temperature changes, a series of responses occur so as to adapt to temperature. For example, expression of specific protein, changes of fatty acid content and corresponding changes of mitochondria. Compared with the 27 °C group, the specific proteins at 5 °C and 10 °C were more than those at 15 °C. This indicated that the more specific proteins took part in the process of lower temperature adaptation. The increase of mitochondria at low temperature indicated that this adaptation required more energy to be produced by the mitochondria. Changes of fatty acid composition also indicated the positive effects of adaptation to a low temperature. All of these changes at low temperature improved the ability of *S. serrata* to cope with temperature changes, so that, as temperature decreased, various changes of biochemistry and physiology were the positive effects of adaptation.

In this study, we also paid attention to of the many non linear changes related to the decrease of temperature, for example, the specific proteins expressed at 5 °C and 10 °C were the same; mitochondria increased significantly only at 10 °C; ΣPUFA-ω3 also increased significantly only at 10 °C. These results imply that different metabolic modes at physiological, biochemical and cellular biology level were used to adapt to low temperature. However, further research must be carried out to prove this viewpoint.

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