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Stearoyl–CoA desaturase expression and fatty acid composition in milkfish (*Chanos chanos*) and grass carp (*Ctenopharyngodon idella*) during cold acclimation

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Abstract

Desaturation of fatty acids is an important adaptation mechanism for fish to maintain membrane fluidity under thermal stress. To comprehend the temperature adaptation mechanism in fish, we investigated the difference in the changes of stearoyl–CoA desaturase expression and fatty acid composition between milkfish and grass carp under cold acclimation. We find that in both fish the proportions of unsaturated fatty acids at 15 °C are all higher than those at 25 °C. In milkfish Δ^9-desaturation index (ratios of 16:1/16:0 and 18:1/18:0) increases significantly in the beginning of cold acclimation at 15 °C and decreases afterward, but in grass carp it increases slightly in the beginning of cold acclimation followed by a sustained dramatic increase. Similarly, activity of stearoyl–CoA desaturase in milkfish increases significantly in the beginning, peaks at day 4, and then decreases constantly, but in grass carp it increases gradually in the first week, rises dramatically afterward, and then maintains a very high level. The change of stearoyl–CoA desaturase activity is parallel to the change of Δ^9-desaturation index in both milkfish and grass carp, but it is one day earlier than Δ^9-desaturation index in milkfish. The difference of adaptation capability between milkfish and grass carp under cold stress is further evidenced by RT-PCR and Northern blot analysis of stearoyl–CoA desaturase gene expression.

Keywords: Acclimation; Desaturation index; Desaturase; Gene expression; Fatty acid; Cold; Milkfish; Grass carp

1. Introduction

Life of ectothermal teleosts is crucially influenced by temperature, including their survival, growth, and reproduction. To maintain physiological homeostasis under severe thermal stress, every teleostean species has developed its own specific adaptative mechanisms, either behavioral, physiological, or biochemical, to cope with temperature fluctuations (Crawshaw and Hammel, 1974; Cossins et al., 1977; Cossins and Bowler, 1987; Crockford and Johnston, 1990; Carey and Scharold, 1991; Prosser and Heath, 1991). However, when severe cold fronts approach in winter, mass mortality of warm-water cultured teleosts still often occurs and causes tremendous losses for the aquaculture industries in Taiwan (Liao, 1991). Consequently, studies on cold-adaptation mechanisms of fish would be very valuable to the progress of the aquaculture industry and fisheries science.

Maintenance of proper membrane fluidity is an important adaptative process for fish during temperature fluctuations. Since unsaturated fatty acids are key components of cellular membranes involved in energy metabolism, alteration of unsaturated fatty acid composition to maintain cell membrane fluidity has been shown to be a key mechanism in the physiological compensation to thermal stress in living organisms (Cossins and Bowler, 1987; Wodtke and Cossins, 1991). Accordingly, for ectothermal teleosts to maintain physiological homeostasis under cold stress, increasing membrane fluidity through an increase in the proportion of

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unsaturated fatty acids has been widely observed in cell membranes (Cossins and Macdonald, 1986; Cossins and Bowler, 1987; Wodtke and Cossins, 1991), such as in common carp (*Cyprinus carpio*.) (Schünke and Wodtke, 1983; Wodtke and Cossins, 1991; Tiku et al., 1996; Trueman et al., 2000) and rainbow trout (*Salmo gairdneri*) (Hazel, 1979; Hagar and Hazel, 1985a,b; Bowden et al., 1996). Desaturation of membrane fatty acids has therefore been widely considered an important adaptative mechanism for fish under cold stress.

In the biosynthesis of monounsaturated fatty acids, stearoyl–CoA desaturase (SCD, EC 1.14.99.5) is the rate-limiting enzyme that introduces the first double bond between the C9 and C10 positions in palmitoyl–CoA (16:0) and stearoyl–CoA (18:0), which are then converted to palmitoleoylCoA (16:1) and oleoylCoA (18:1), respectively (Jeffcoat et al., 1977; Ntambi, 1995; Tocher et al., 1998). Since stearoyl–CoA desaturase has strong impacts on the desaturation of 16:0 and 18:0 fatty acids, activation and intensification of stearoyl–CoA desaturase activity at a low temperature would lead to significant increases in the proportion of unsaturated fatty acids, as indicated by Δ^9 desaturation index (ratios of 16:1/16:0 and 18:1/18:0), thereby enhancing the adaptation capability of fish under cold acclimation.

Our previous studies showed that expression of stearoyl–CoA desaturase is tissue-specific in grass carp (*Ctenopharyngodon idella*) (Chang et al., 2001) and detectable in zebrafish (*Danio rerio*) during embryogenesis and early development (Hsieh et al., 2003). It can be induced by cold stress in milkfish (*Chanos chanos*) and stimulated by hormonal treatment in tilapia (*Oreochromis mossambicus*) (Hsieh et al., 2004). To comprehend the temperature adaptation mechanism in fish and to compare differences between stenothermal and eurythermal teleosts, we investigated the time-course changes of stearoyl–CoA desaturase expression and fatty acid composition in milkfish (*C. chanos*) and grass carp (*C. idella*) under cold acclimation. Milkfish are an economically important cultured species in the Southeast Asian region, but they have been reported to experience severe stress and high mortality when the water temperature drops below 15 °C (Chen, 1990; Liao, 1991). Grass carp are a kind of euryothermal teleost capable of tolerating wide-ranging temperature fluctuations by maintaining physiological homeostasis through inherited regulatory capabilities. This paper presents the results of comparisons of physiological and biochemical responses between milkfish and grass carp under cold acclimation for various times.

2. Materials and methods

2.1. Fish

Milkfish (*C. chanos*) were collected from the Tainan Station of the Taiwan Fisheries Research Institute and grass carp (*Ctenopharyngodon idella*) were obtained from a local farm. Their average body masses were 66.5 ± 4.3 and 48.5 ± 1.3 g, respectively. Before the experiments, fish in both the control and cold-treatment groups were all acclimated to fresh water at 25 °C in an environment-controlled room under a 12 L/12 D photoperiod regime and fed commercially formulated fish feed. All fish were starved for 24 h before the experiment was initiated. After the water temperature for the cold-treatment groups reached 15 °C, all fish were fed as usual.

2.2. Experimental design and sampling

Prior to the cooling treatment, five fish of each species were sampled as an initial control. The water temperature for the cold-treatment groups was then changed from 25 to 15 °C at a rate of 0.5 °C/h by a refrigerated cooler (Firstek, B403L, UK). After the water temperature for the cold-treatment groups reached 15 °C, five of milkfish and grass carp each from the cold treatment (15 °C) and control (25 °C) groups were sampled daily for a week. After day 8, all the milkfish had died, but the grass carp were still sampled on days 14 and 21. For each sampling, five fish were sacrificed and the livers were rapidly removed and stored at −80 °C for later isolation of hepatic microsomes.

2.3. Fatty acid composition analysis

Microsomes were prepared following methods described by Hagar and Hazel (1985a). All procedures were performed at 0–4 °C. Liver tissues were weighed, minced, and homogenized in six volumes (w/v) of homogenizing buffer (25 mM sucrose, 10 mM Tris–acetate (pH7.8), containing 1 mM EDTA, and 1.5 mM glutathione) with a Teflon homogenizer. The homogenate was centrifuged at $10{,}000 \times g$ and 4 °C for 10 min. The pellet was resuspended in 60 mM Tris–acetate (pH 7.0) containing 1.5 mM glutathione and immediately frozen at −80 °C for analyses of stearoyl–CoA desaturase activity and fatty acid composition. Lipids were extracted from hepatic microsomes according to the method described by Bligh and Dyer (1959). The extracted lipids were subjected to methanolysis in 5% HCl in methanol at 85 °C for 2.5 h (Christie, 1982). The resultant fatty acid methyl esters were analyzed with a gas chromatograph (Shimadzu, GC-14A, Japan) equipped with a flame ionization detector. The chromatograph was programmed with an initial holding temperature at 140 °C for 5 min, ramping at 5 °C/min to 220 °C, and a final holding temperature at 220 °C for 7 min. The injector temperature was 250 °C and the temperature of the flame ionization detector was maintained at 260 °C. Samples were analyzed using a 5-mm ID glass column packed with 15% DEGS (diethyleneglycol succinate polyester, SP 2330, 60 m × 0.25 mm). The relative fatty acid content was determined by comparing

peak areas with that of the internal standard, heptadecanoic acid (Sigma).

2.4. Stearoyl–CoA desaturase activity analysis

Stearoyl–CoA desaturase activities of hepatic microsomes were measured spectrophotometrically (Hitachi, U-2000 spectrophotometer) by monitoring NADH-induced reduction and stearoyl–CoA-stimulated reoxidation of cytochrome b_5 at 424 and 409 nm (Oshino and Sato, 1972; Schünke and Wodtke, 1983). The reaction mixture contained 100 mM Tris–HCl (pH 7.2), 200 μl freshly prepared Na_2S, and 1 mg microsomal protein in a final volume of 1 ml. The rate of stearoyl–CoA-stimulated oxidation of reduced cytochrome b_5 was calculated as described by Schünke and Wodtke (1983).

2.5. Isolation of total RNA and reverse transcription-polymerase chain reaction (RT-PCR)

RNA was isolated from frozen tissue using an Ultraspec™-II RNA isolation system (Biotecx Laboratories, USA) following the manufacturer's instructions. First-strand cDNA was synthesized using 5 μg of total RNA isolated from the liver, MMLV reverse transcriptase (Promega, Madison, WI, USA), and 50 ng of oligo (dT) primer for 1 h at 37 °C. Two primers specific to milkfish desaturase were used: 5′-ACACT TTCCT TGGAC AAATT GTGTG-3′ (sense strand) and 5′-AGCCA GGTCG CGTTA AGGAC-3′ (antisense strand) (Hsieh et al., 2001). The grass carp desaturase-specific primers used for PCR were: 5′-CCATC TGCAT CTTTT CTCAC-3′ (sense strand) and 5′-TTACG GACCA ACAGC CAGCC-3′ (antisense strand) (Chang et al., 2001). The PCR reaction conditions included an initial step at 94 °C for 3 min, followed by 30 cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min. A final extension step was performed at 72 °C for 6 min. The amplified product was electrophoresed in a 1.2% (w/v) agarose gel.

2.6. Northern blot analysis

RNA (10 μg) was separated on a 1% agarose gel in the presence of 3-(*N*-morpholino) propanesulfonic acid (MOPS) and formaldehyde (Sambrook et al., 1989). The RNAs were then transferred to Hybond N+ nylon membrane (Schleicher and Schuell, USA) and fixed using UV irradiation cross-linking (Stratagene, La Jolla, CA, USA). The hybridization DNA probe prepared was radioactively labeled with (^{32}P) dCTP (random primer kit, Stratagene) and used as a hybridization probe. The ^{32}P-labeled DNA probe (containing 1×10^6 cpm/ml) was hybridized with the membrane at 42 °C overnight. After hybridization, the blot was washed once in 0.2X wash buffer (0.2X SSC, 0.1% SDS) at 42 °C, and twice in 0.1X wash buffer (0.1X SSC, 0.1% SDS) at 42, 50 and 68 °C for 15 min, respectively. The membranes were exposed to PhosphorImager (Molecular Dynamic, OR, USA) for radioactivity analysis.

2.7. Statistical analyses

Data are presented as the mean ± SEM for five fish. Significant differences between milkfish and grass carp at the same time point were tested using one-way analysis of variance (ANOVA). A value of $p<0.05$ was used to indicate statistical significance.

3. Results

3.1. Fatty acid composition

Changes in the fatty acid composition of hepatic microsomes of milkfish and grass carp after transfer from 25 to 15 °C are shown in Fig. 1. The proportion of saturated fatty acids in milkfish steadily decreased from 76.5 ± 4.39% to 13.4% ± 1.63% within the first 5 days and then increased slightly on days 6 and 7. In grass carp, however, the proportion of saturated fatty acids was maintained between 52.6 ± 2.7% and 57.4 ± 2.5% without significant changes for the first 7 days but had visibly decreased to 49.1 ± 4.3% and 36.5 ± 1.3% on days 14 and 21, respectively (Fig. 1A).

Contrarily, the proportion of unsaturated fatty acids in milkfish steadily increased in the beginning and then slightly decreased. In milkfish, the proportions of monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) peaked on days 5 and 3, respectively. The proportion of PUFAs in milkfish increased from 7.17 ± 0.75% to 31 ± 3.17% 3 and then had returned to a value similar to the control on days 6 and 7. In grass carp, however, the proportions of MUFAs and PUFAs showed no significant changes within the first 7 days, but both had obviously increased on days 14 and 21. The proportion of PUFAs in grass carp on day 21 was double the value of the control (Fig. 1B and C).

3.2. Activity of stearoyl–CoA desaturase

Fig. 2 shows changes in stearoyl–CoA desaturase activities in hepatic microsomes of milkfish and grass carp during acclimation from 25 to 15 °C. In milkfish, the desaturase activity for the control group was maintained at between 1.19 ± 0.18 and 1.32 ± 0.16 nmole/min/mg protein during the experiment, but the activity for the cold-treatment group increased 1.4-fold to 3.08 ± 0.24 nmole/min/mg protein on day 4, followed by a steady decrease to 1.82 ± 0.24 nmole/min/mg protein on day 7. The activity for the cold-treatment group at 15 °C was always higher than that for the control group at 25 °C within the 7-day period of acclimation.

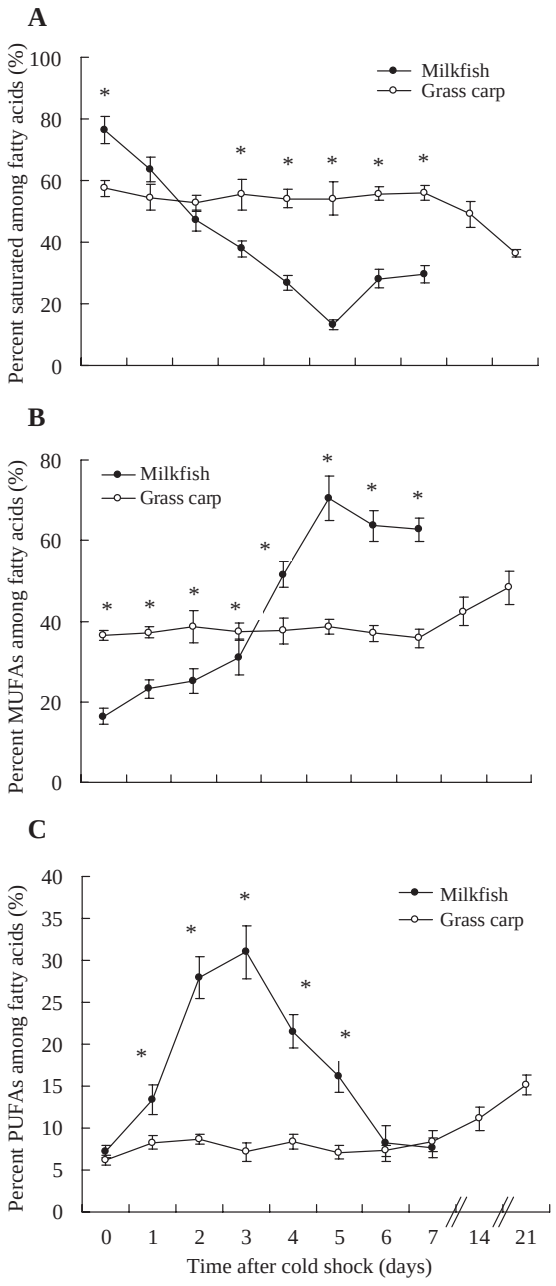

Fig. 1. Changes in the fatty acid composition of saturated (A), monounsaturated (B), and polyunsaturated fatty acids (C) in liver microsomes of milkfish (solid circles) and grass carp (open circles) after transfer from 25 to 15 °C. Values represent the mean ± SEM (n=5). Asterisks (*) indicate values which significantly differ from the respective control values on day 0 at the 5% level (by Student's t-test).

In grass carp, the desaturase activity for the control group was maintained at between 1.02 ± 0.26 and 1.32 ± 0.23 nmole/min/mg protein. For the cold-treatment group, it increased slightly but was maintained in the range of 1.45 ± 0.23–1.89 ± 0.10 nmole/min/mg protein. But it had significantly increased to 2.33 ± 0.11 nmole/min/mg protein on day 14 and to 3.86 ± 0.27 nmole/min/mg protein on day 21. Like that in milkfish, the desaturase activity in grass carp for the cold-treatment group at 15 °C was also always higher than that for the control group at 25 °C within the 21-day period of acclimation.

3.3. Correlation of stearoyl–CoA desaturase activity and the desaturation index

Changes in stearoyl–CoA desaturase activity and the desaturation index of hepatic microsomes of milkfish and grass carp during acclimation from 25 to 15 °C are shown in Fig. 3. In milkfish, the Δ^9 desaturation index (ratios of 16:1/16:0 and 18:1/18:0) initially at 25 °C was 0.15 and 0.5, respectively. During acclimation at 15 °C, value significantly increased to 3.18 and 4.98 correspondingly on day 5, followed by considerable decreases on days 6 and 7. Furthermore, in milkfish the change in the Δ^9 desaturation index is parallel to or was 1 day later than the change in stearoyl–CoA desaturase activity. The Δ^9 desaturation index peaked on day 5, while the desaturase activity peaked on day 4. Both of them decreased steadily after the peak. In grass carp, however, the Δ^9 desaturation index showed no significant changes within the first 6 days but steadily and dramatically increased after day 7. During acclimation at 15 °C, the change in Δ^9 desaturation index in grass carp was also more significant than the change in desaturase activity, but it occurred 1 day earlier rather than 1 day later in milkfish.

3.4. Stearoyl–CoA desaturase expression

Expressions of stearoyl–CoA desaturase mRNA and time-course changes in desaturase transcripts in milkfish and grass carp under after transfer from 25 to 15 °C were investigated by RT-PCR and Northern blot analysis of total RNA (Fig. 4). The Northern blot analysis showed that expression of stearoyl–CoA desaturase mRNA in milkfish

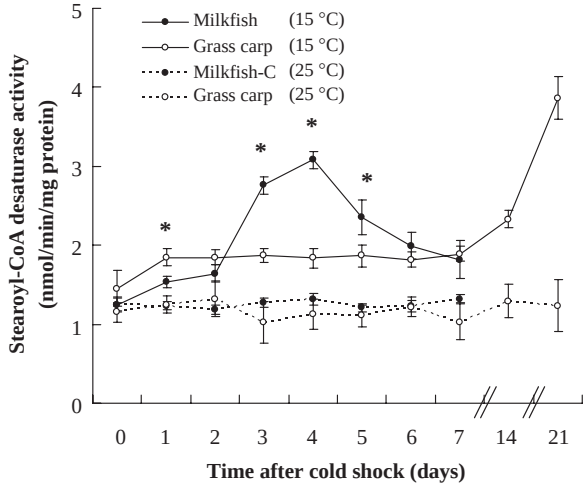

Fig. 2. Time-course changes in stearoyl–CoA desaturase activity in hepatic microsomes of milkfish (solid circles) and grass carp (open circles) after transfer from 25 (dotted line) to 15 °C (solid line). Values represent the mean ± SEM (n=5). Asterisks (*) indicate mean values that significantly differ between milkfish and grass carp at the same time point (p<0.05).

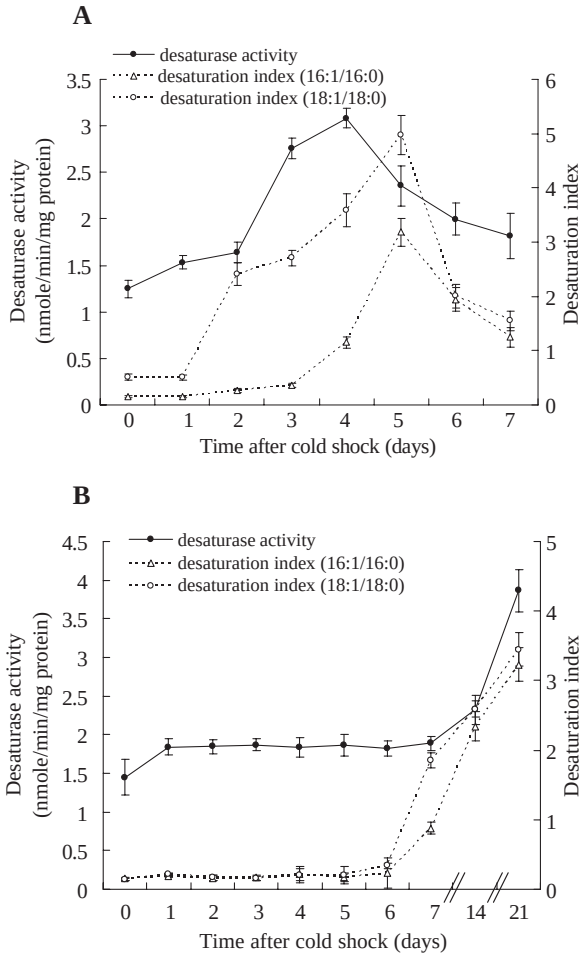

Fig. 3. Changes in the desaturase index (16:1/16:0 and 18:1/18:0 open triangles and circles, respectively) and stearoyl–CoA desaturase (solid circles) in liver microsomes of milkfish (A) and grass carp (B) after transfer from 25 to 15 °C. Values represent the mean ± SEM (n= 5).

was weak at 25 °C but, during acclimation to 15 °C, it steadily increased within the first 5 days, but then significantly decreased again and was undetectable by day 7. Consistently, RT-PCR also showed that expression of stearoyl–CoA desaturase mRNA in milkfish was abundant during the first 5 days of cold acclimation (Fig. 4A, B). On the other hand, the transcription of stearoyl–CoA desaturase mRNA was only strongly detectable in grass carp on day 21; neither Northern blot analysis nor RT-PCR was detectable within the first 14 days of acclimation at 15 °C (Fig. 4D, E).

4. Discussion

Considering changes in the fatty acid composition of liver microsomes under cold acclimation, our results show that the proportions of saturated fatty acids in milkfish and grass carp at 15 °C were all lower than those at 25 °C, but the proportions of unsaturated fatty acids at 15 °C were all higher than those at 25 °C. This is evidence that desaturation of membrane fatty acids as observed in both stenothermal and eurythermal teleosts like milkfish and grass carp used to increase the proportion of unsaturated fatty acids during cold acclimation. Further, the proportions of monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) changed differently in milkfish and grass carp. In milkfish, PUFA initially increased and then decreased right back to the low level they were initially at 25 °C, but MUFA did not drop significantly after considerable elevation. The level of PUFA might be increased through the elongation of MUFA (Stoffel and Ach, 1964). Previous studies showed that PUFA biosynthesis might initially be chain elongated, followed by desaturation, while β-oxidation process might be also involved in the change of PUFA level. These

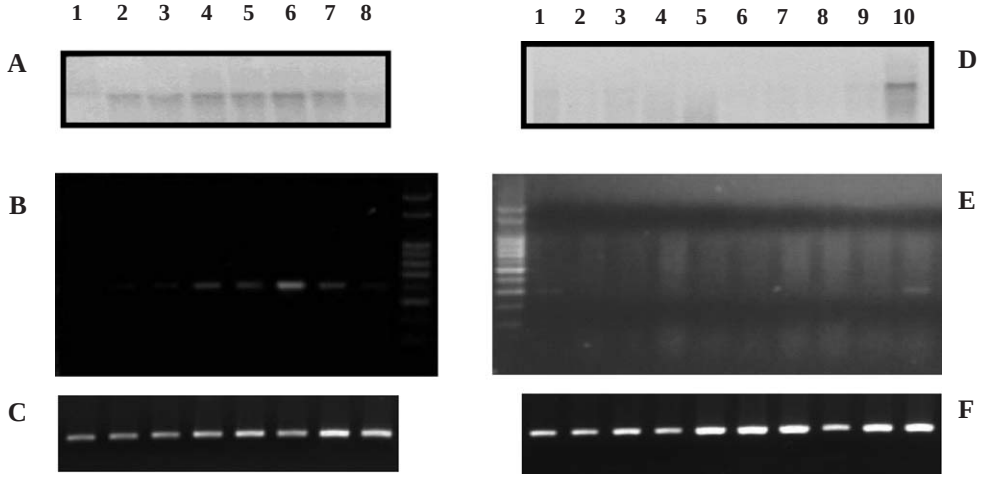

Fig. 4. Northern blot and RT-PCR analyses of the expression of milkfish (A–C) and grass carp (D–F) desaturase at different times under cold shock (milkfish: lanes 1–8; 1: 0, 2: 1, 3: 2, 4: 3, 5: 4, 6: 5, 7: 6, and 8: 7 days and grass carp: lanes 1–11; 1: 0, 2: 1, 3: 2, 4: 3, 5: 4, 6: 5, 7: 6, 8: 7, 9: 14, and 10: 21 days). Desaturase mRNA levels were determined by Northern blot analysis in milkfish (A) and grass carp (D). For each sample, total RNA at 10 μg was loaded for each lane. The blot was the result of hybridization with a DNA probe. The expression of stearoyl–CoA desaturase mRNA was also analyzed by RT-PCR with primers specific to milkfish (B) and grass carp (E) stearoyl–CoA desaturase. The PCR products were analyzed in a 1.2% gel with a DNA marker. The internal controls (β-actin) for the RT-PCR are shown in C and F.

processes may be involved in the big increase in PUFA for milkfish under severe cold stress (Sprecher, 2000).

The activity of stearoyl–CoA desaturase significantly increases and is correlated to membrane fluidity during thermal compensation (Trueman et al., 2000). Similar to changes in the proportion of unsaturated fatty acids during cold acclimation, the activity of stearoyl–CoA desaturase in milkfish significantly increased in the beginning, peaked on day 4, and then continually decreased until exhaustion. There may be acute disruption in thermal regulation or metabolism in milkfish around day 4 under long-term cold stress. In grass carp, however, the activity of stearoyl–CoA desaturase gradually increased in the first week, rose dramatically afterward, and was maintained at a very high level on day 21. Similar results can be found in rainbow trout, in which the activity of stearoyl–CoA desaturase also increases late and remains at a very high level (Hagar and Hazel, 1985a). In common carp, the activity of stearoyl–CoA desaturase also rapidly increased in the beginning, but the magnitude of the change in common carp, nearly a 30-fold increase, was much stronger than that in milkfish, at only a 1.4-fold increase. We found that the activity of stearoyl–CoA desaturase in fish always increased during cold acclimation, but the time-course of changes greatly differed between species depending on their capability of evolutionarily inherited cold tolerance.

Stearoyl–CoA desaturase plays an important role in the metabolism of fatty acids and regulation of membrane fluidity under temperature fluctuations (Jeffcoat, 1979; Tiku et al., 1996; Vigh et al., 1993). During long-term low temperatures, the proportion of unsaturated fatty acids in cell membranes of common carp increases as does the activity of stearoyl–CoA desaturase (Schünke and Wodtke, 1983), which is induced by transduction of the stearoyl–CoA desaturase gene (Trueman et al., 2000). In rainbow trout, changes in the proportion of monounsaturated fatty acids also parallel changes in stearoyl–CoA desaturase under temperature fluctuations (Hagar and Hazel, 1985a,b). To quantify the correlation between the activity of stearoyl–CoA desaturase and desaturation of fatty acids, we further analyzed the time-course changes of stearoyl–CoA desaturase activity and the Δ^9 desaturation index. As shown in Fig. 3, correlations between the changes in stearoyl–CoA desaturase activity and the 16:1/16:0 ratio in milkfish and grass carp were 0.88 and 0.90, respectively, and correlations between the changes of stearoyl–CoA desaturase activity and the 18:1/18:0 ratio were 0.81 and 0.84, respectively. These results suggest that changes in stearoyl–CoA desaturase activity parallel those of the Δ^9 desaturation index in both milkfish and grass carp. However, the change in stearoyl–CoA desaturase activity occurred 1 day earlier than that of the Δ^9 desaturation index in milkfish, but they changed concurrently in grass carp. In milkfish, stearoyl–CoA desaturase activity peaked on day 4, but the Δ^9-desaturation index peaked on day 5. This may have resulted from the synthesis and decomposition of stearoyl–CoA desaturase enzyme in milkfish under cold acclimation being less active or efficient than those in grass carp. This may explain why grass carp has better adaptability to thermal stress than milkfish.

The effects of PUFA on stearoyl–CoA desaturase gene could be at the levels of transcript and mRNA stability (Sessler et al., 1996; Gonzalez and Martin, 1996; Waters et al., 1997). Expression of the stearoyl–CoA desaturase gene in milkfish and grass carp under cold acclimation was further investigated by RT-PCR and Northern blot analysis. We found that expression of stearoyl–CoA desaturase mRNA was induced in both milkfish and grass carp under cold shock, but the time-course of changes in stearoyl–CoA desaturase transcripts differed between milkfish and grass carp. Transcripts of stearoyl–CoA desaturase mRNA were detectable in milkfish from day 2 of cold acclimation, but they began to decrease after day 5 and were undetectable on day 7. In grass carp, however, transcripts of stearoyl–CoA desaturase mRNA were detectable on day 21, after a long period of cold acclimation. Therefore, cold stress did impact the expression and transcription of stearoyl–CoA desaturase mRNA in both stenothermal and eurythermal teleosts, although there were differences in the time-course of changes in expression.

Many factors might simultaneously be involved in the process of physiological compensation and thermal adaptation of fish, such as energy metabolic enzymes and cold shock proteins (Jones et al., 1996). Our study reveals that there were significant changes and differences in stearoyl–CoA desaturase expressions and fatty acid compositions between milkfish and grass carp under cold acclimation from 25 to 15 °C. Changes in stearoyl–CoA desaturase activity paralleled those of the Δ^9 desaturation index. During cold acclimation at 15 °C, milkfish became comatose or died after day 8, but grass carp still survived even after exposure to 21 days of low temperatures. This difference in the capability for cold tolerance between stenothermal and eurythermal teleosts is well demonstrated by the changes in stearoyl–CoA desaturase expression and fatty acid composition as revealed by our experiments on milkfish and grass carp under cold acclimation.

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