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Extending the pre-rigor state of fish by enhancing mitochondrial ATP synthesis

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Abstract

In Japan, spiked fish in a pre-rigor state have extremely high commercial value, similar to that of live fish. It has been claimed that the progress of rigor mortis is slower in carp acclimated to 10°C than in fish acclimated to 30°C during storage at either 0°C or 10°C. Wild specimens of plaice captured from cold waters in winter also show retarded rigor-mortis progress at 10°C compared with fish captured from warm water in other seasons.

Two factors are supposed to be associated with such temperature-dependent rigor-mortis progress in fish. One is intracellular Ca^{2+} concentration and myofibrillar adenosine triphosphate (ATP) consumption—enhanced by the increase of Ca^{2+}, which is released from the sarcoplasmic reticulum (SR; the Ca^{2+}-storing organelle in muscle) via nervous stimuli. Ca^{2+} uptake by SR is very low at 0°C due to a low activity of Ca^{2+}-ATPase for the Ca^{2+} pump. Subsequently, fish rigor mortis is accelerated at 0°C due to the increase of intracellular Ca^{2+} concentration. The other factor is ATP synthesis in mitochondria. We have recently found that the content of mitochondrial ATP synthase (FoF1-ATPase) was higher in carp acclimated to 10°C than in fish acclimated to 30°C. Oligomycin-sensitive FoF1-ATPase activity per mitochondrial protein weight was three times higher in carp acclimated to 10°C than 30°C. Furthermore, plaice and red seabream adapted to about 10°C contained larger quantities of ATP synthase than fish acclimated to about 25°C, irrespective of species. These changes suggest that alterations in FoF1-ATPase, functioning at the final step of ATP production, are related to the progress of rigor mortis in fish.

Introduction

COMMERCIALLY valuable bottom fish, such as seabream and plaice, require extremely high freshness to be consumed raw and, in Japan, are often distributed to the market alive. However, if these fish can be supplied to the market in pre-rigor state, there is no need to transport them alive, since pre-rigor fish have the same high commercial value as live specimens. Furthermore, one of the *umami* taste compounds, inosinic acid (IMP)—first discovered from dried skipjack tuna (*Katsuowonus pelamis*) in Japan in terms of a taste component—greatly accumulates in fish muscle within a certain period, but not immediately, after death. Therefore, rather than keeping fish alive through the marketing period, which is time and energy consuming, they should be stored in pre-rigor state, maximizing the IMP accumulation when eaten raw.

Rigor mortis is one of the prominent changes in muscle occurring soon after death. The related biochemistry has been studied extensively in fish (Iwamoto et al. 1987, 1988; Watabe et al. 1989, 1990, 1991). When fish are killed while relaxed, creatine phosphate is degraded before destruction of adenosine triphosphate (ATP). ATP content starts decreasing when the creatine phosphate level reaches about the same concentration as ATP. Lactate accumulation proceeds parallel to ATP degradation and the degree of rigor mortis. Such postmortem changes in fish muscle are retarded when fish are killed by cranial spiking while alive. Furthermore, it has been demonstrated that in temperate fish—such as red seabream (*Pagrus major*), plaice (*Paralichthys olivaceus*) and carp (*Cyprinus carpio*)—the rate of ATP degradation is clearly slower at 5–15°C than at 0°C, resulting in retar-

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dation of rigor-mortis onset at the former temperatures (Iwamoto et al. 1985, 1987, 1988; Watabe et al. 1991). Two factors are supposed to be associated with such temperature-dependent rigor-mortis progress in fish. It is strongly suggested that the acceleration of temperate fish rigor mortis at 0°C is due to an increase in Ca^{2+} concentration in muscular cells during storage at this temperature, because Ca^{2+} uptake by the sarcoplasmic reticulum (SR), the Ca^{2+}-storing organelle in muscle, decreases with the decrease in reaction temperature (Watabe et al. 1989; Ushio et al. 1991).

Besides Ca^{2+} concentrations in muscular cells, the other factor which may control ATP degradation after death, and thus to be associated with the progress of rigor mortis, is ATP production in mitochondria via ATP synthase. We have recently found that the content of mitochondrial ATP synthase (FoF1-ATPase) is higher in carp acclimated to 10°C than in fish acclimated to 30°C (Kikuchi et al. 1999; Itoi et al. 2003). It has been also claimed that wild specimens of plaice captured from winter cold waters show retarded rigor-mortis progress at 10°C compared with fish captured from warm water in other seasons (Tanaka 1991). Furthermore, the state of fish rigor was apparently reflected in the market price irrespective of species (plaice or red seabream) (Tanaka 1991). Thus, we examined the changes in mitochondrial ATP synthase in red seabream and plaice acclimated to low and high temperatures. Fish acclimated to about 10°C contained larger quantities of ATP synthase than fish acclimated to about 25°C, irrespective of species (S. Itoi et al., unpublished data).

Regulation of rearing conditions of fish by environmental physical factors, such as temperature, which may increase mitochondrial ATP synthase, appears to be an effective way of retarding rigor-mortis progress of spiked fish, thereby resulting in the increase of commercial value. This paper deals with the possibility of retarding the progress of fish rigor mortis by enhancing ATP production via mitochondria.

Fish rigor-mortis progress and its dependence on storage temperature

IWAMOTO et al. (1987) tested for changes in rigor tension and ATP degradation in the muscle of live plaice specimens when spiked in the brain and stored at various temperatures ranging from 0°C to 20°C. The ATP degradation rate was clearly slower at 5–15°C than at 0°C, resulting in retardation of rigor-mortis onset at the former temperatures. Lactic acid accumulation in the muscle correlated well with the decrease of ATP. Fish stored at 10°C, for example, showed an ATP concentration of 4 μmol/g and 'rigor index' of 20% (full rigor = 100%) when lactic acid increased up to 25 μmol/g (Figure 1). The muscle reached full rigor when ATP almost completely disappeared and lactic acid attained the maximum plateau (40–50 μmol/g). ATP concentration was constant at 5 μmol/g until creatine phosphate decreased from the initial concentration at around 20 μmol/g to 5 μmol/g, irrespective of storage temperature (Iwamoto et al. 1988). Simultaneously, lactic acid accumulated—slowly at first, until ATP concentration started decreasing, and then quickly, accompanying the full-rigor state.

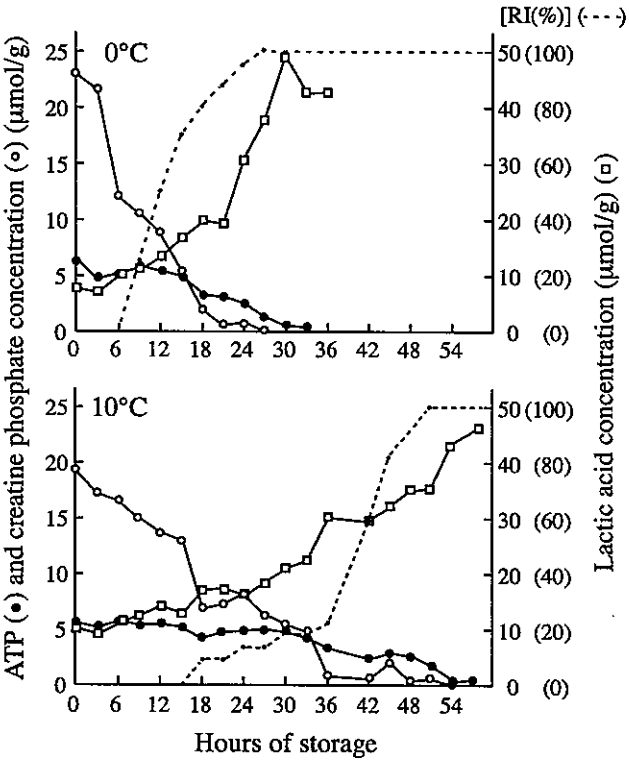

Figure 1. Rigor-mortis progress in plaice stored at 0°C and 10°C in relation to postmortem, biochemical changes (RI = rigor index, where 100% represents full rigor). Modified from Iwamoto et al. (1988).

In order to elucidate the mechanisms involved in the acceleration of fish rigor mortis at 0°C, the temperature-dependency of activity of some enzymes involved, such as myofibrillar and sarcoplasmic ATPases and creatine kinase, were investigated for plaice (Iwamoto et al. 1988). These enzymes all decreased with the decrease in reaction temperature. While it is assumed that spiked fish muscle is in a relaxed state and the intracellular Ca^{2+} concentration is low, the progress rate of rigor mortis at 0°C correlated well with Mg^{2+}-ATPase activity, a physiologically important issue for ATP consumption, in the presence of a few micromolar Ca^{2+} at 0°C (Watabe et al. 1989). Correspondingly, it was found that Ca^{2+} uptake by the sarcoplasmic reticulum (SR) of plaice was extremely low at 0°C due to a low activity of Ca^{2+}-ATPase for the Ca^{2+} pump (Figure 2). Taken together, it is strongly suggested that the acceleration of fish rigor mortis at 0°C is due to the

increase of intracellular Ca^{2+} concentration during storage at this temperature.

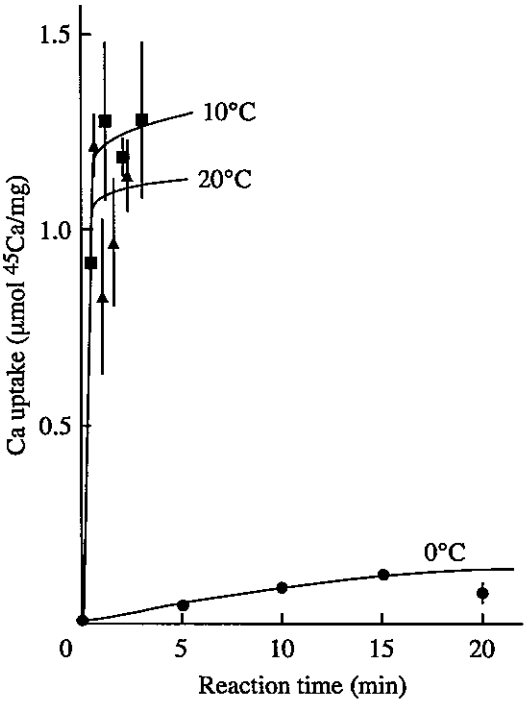

Figure 2. Ca^{2+}-uptake by the sarcoplasmic reticulum of plaice at 0, 10 and 20°C. Bars represent means ± standard errors for three measurements. Modified from Watabe et al. (1989).

Changes in fish muscle proteins in association with acclimation temperature

WIDE changes in body temperature are experienced seasonally by poikilotherms, especially eurythermal temperate zone fish, such as carp and goldfish (*Carassius auratus*). Watabe et al. (1990) and Hwang et al. (1991) acclimated carp to 10°C and 30°C and examined the progress of rigor mortis, along with ATP and creatine phosphate degradation and lactate accumulation, during storage at 0, 10, and 20°C. Rigor-mortis progress and related biochemical changes were slower with the cold-acclimated than with warm-acclimated carp during storage at 0°C or 10°C, and vice versa at 20°C. Figure 3 shows postmortem changes of carp acclimated to either 10°C or 30°C as examples (Watabe et al. 1990). It is apparent that carp acclimated to 10°C had retarded rigor-mortis progress compared to fish acclimated to 30°C when stored at 0°C after spiking.

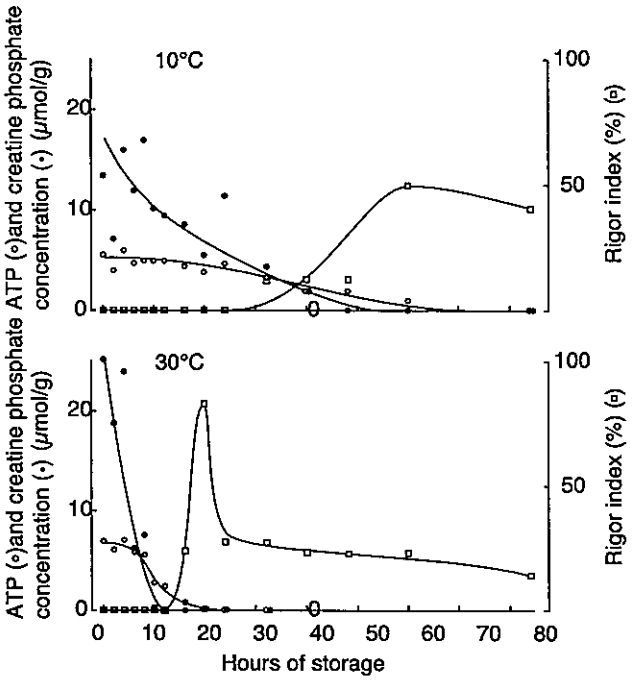

Figure 3. Changes in rigor-mortis progress in carp related to acclimation temperature. Modified from Watabe et al. (1990).

To elucidate the mechanisms underlying differences in rigor-mortis progress between cold- and warm-acclimated carp, SR Ca^{2+} uptake rate was compared *in vitro* at a reaction temperature of 0°C (Watabe et al. 1990) (Table 1). The rate for cold-acclimated carp was about twice as high as that for warm-acclimated carp. Therefore, rigor-mortis acceleration in warm-acclimated carp during storage at 0°C is probably again due to its poor SR Ca^{2+} uptake ability. Ushio and Watabe (1993) claimed that carp modify both the fluidity of SR membranes and the molecular structure of Ca^{2+}-ATPase following temperature acclimation to compensate for fluctuating ambient temperatures.

Table 1. Ca^{2+}-uptake rate and myofibrillar Mg^{2+}-ATPase activity in relation to temperature acclimation of carp.

Acclimation temperature	Sarcoplasmic reticulum Ca^{2+}-uptake rate (nmol Ca/min.mg)		Myofibrillar Mg^{2+}-ATPase activity (μmol P_i/min.mg)	
	+Oxalate	–Oxalate	+Ca	–Ca
10°C	7.17 ± 0.83	6.23 ± 0.72	0.130 ± 0.037	0.003 ± 0.001
30°C	5.81 ± 1.20	3.32 ± 0.67	0.050 ± 0.004	0.003 ± 0.001

Notes: Ca^{2+}-uptake rate and Mg^{2+}-ATPase activity were measured at 0°C. Data are given as mean ± standard error for three measurements. Modified from Watabe et al. (1990).

It has been also shown that carp myofibrillar Mg^{2+}-ATPase activity in the absence of Ca^{2+}, reflecting the conditions for spiked fish, is the same for both cold- and warm-acclimated carp (Hwang et al. 1990; Watabe et al. 1990) (Table 1). However, cold-acclimated carp showed about three times the activity in the presence of Ca^{2+}. Given that the same concentration of Ca^{2+} is present in both muscles, cold-acclimated carp should deplete ATP more quickly, thus accelerating the progress of rigor mortis. The results obtained were, however, opposite. Therefore, it seems that Ca^{2+} uptake rate of SR has more effect on the determination of rigor-mortis progress for fish stored at 0°C. Alternatively, another factor(s) may be further responsible for compensating for the effect of myofibrillar Mg^{2+}-ATPase activity in the presence of Ca^{2+} at 0°C enhanced by cold acclimation.

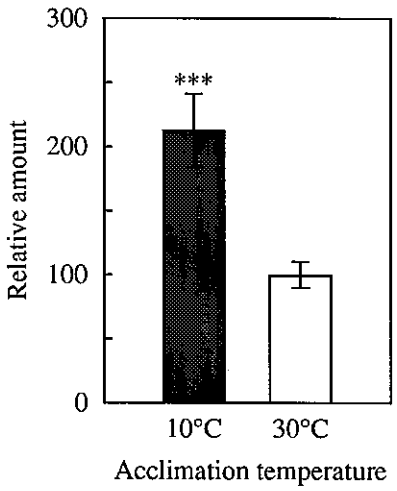

Figure 4. The content of β-F1-ATPase in fast skeletal muscle of carp acclimated to 10°C or 30°C. The average value (n = 3) of β-F1-ATPase levels for the 30°C-acclimated carp was taken as 100. Bars represent means ± standard deviation (n = 3). Student's t-test was employed for statistical comparison (*** = $P <$ 0.005). Modified from Kikuchi et al. (1999).

Changes in ATP synthase in fish mitochondria in association with acclimation temperature

One of the factors that may influence the rate of ATP depletion during rigor mortis is ATP regeneration in mitochondria. ATP synthase (FoF1-ATPase) in mitochondria harnesses the potential energy of the proton gradient produced by electron transport chain complexes including cytochrome *c* oxidase (COX) to synthesize ATP from adenosine diphosphate (ADP) and inorganic phosphate (P_i) (Senior 1988). The eukaryotic FoF1-ATPase is a very large molecule consisting of two functional components which are structurally well defined: a hydrophilic F1 which contains catalytic sites for ATP synthesis, and a proton channel, Fo, embedded in the mitochondrial inner membrane. F1 is further composed of α-, β-, γ-, δ- and ε-subunits, whereas Fo contains more subunits called a, b, c, d, e, F6, OSCP and A6L (Sangawa et al. 1997). The a and A6L subunits of the Fo domain, that are also collectively called ATPase 6–8, are encoded by mitochondrial genes, whereas all other subunits are distinctly encoded by nuclear genes (Anderson et al. 1981).

We revealed, using two-dimensional electrophoresis, that the 55 kDa protein which increased in carp acclimated to 10°C compared to fish acclimated to 30°C was mitochondrial ATP synthase (FoF1-ATPase) β-subunit (Kikuchi et al. 1999) (Figure 4). We subsequently demonstrated that the accumulated levels of mRNA for FoF1-ATPase subunits encoded by nuclear genes were about two times higher per unit weight of total RNA in carp acclimated to 10°C than those in fish acclimated to 30°C (Itoi et al. 2003) (Table 2, Figure 5). On the other hand, the transcripts of the subunits encoded by mitochondrial genes for the 10°C-acclimated carp were 6–7 times as much as those for the 30°C-acclimated carp. In contrast to the changes of mRNA accumulation, no apparent alteration was

Table 2. Comparison in accumulated levels of mRNAs encoding ATP synthase (FoF1-ATPase) subunits of carp acclimated to 10°C or 30°C.

Gene	Subunit	Acclimation temperature	
		10°C	30°C
Nuclear	α-F1-ATPase	2.08 ± 0.42*	1.00 ± 0.30
	β-F1-ATPase	2.18 ± 0.26***	1.00 ± 0.12
	γ-F1-ATPase	2.28 ± 0.46*	1.00 ± 0.38
	c-Fo-ATPase	1.98 ± 0.17***	1.00 ± 0.17
Mitochondrial	ATPase 6-8	5.85 ± 1.53**	1.00 ± 0.38
	COXII	6.73 ± 2.15*	1.00 ± 0.23
	Cytochrome *b*	7.50 ± 2.43*	1.00 ± 0.32

Notes: The mRNA levels of FoF1-ATPase subunits are shown as mean ± standard deviation (n = 3) with relative values for carp acclimated to 30°C as 1.00. Differences in mRNA levels are significant between carp acclimated to 10°C and 30°C at $P < 0.005$ (***), $P < 0.01$ (**), and $P < 0.05$ (*). Modified from Itoi et al. (2002a).

observed in mitochondrial protein composition between carp acclimated to 10°C and 30°C (Figure 6). However, oligomycin-sensitive FoF1-ATPase activity per mitochondrial protein weight was almost three times higher in the 10°C- than 30°C-acclimated carp (Figure 7). These changes suggest that alterations in FoF1-ATPase, functioning at the final step of ATP production, are related to rigor-mortis progress of carp.

Wild specimens of plaice captured from cold waters in winter show retarded rigor-mortis progress at 10°C compared with fish captured from warm water in other seasons (Tanaka 1991) (Table 3). Furthermore,

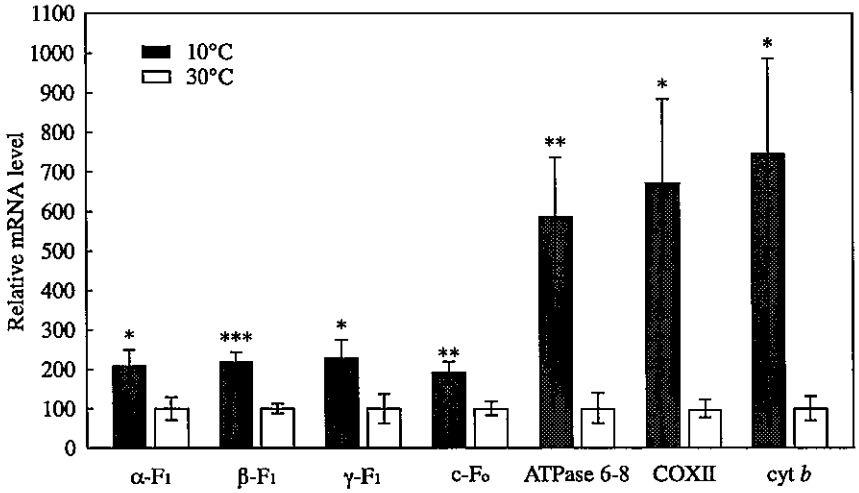

Figure 5. The mRNA levels of FoF1-ATPase subunits and other mitochondrial genes encoded in fast skeletal muscle of carp acclimated to 10°C or 30°C. Bars represent means ± standard deviation (n = 3). Student's t-test was employed for statistical comparison (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.005$). Modified from Itoi et al. (2002a).

Table 3. Evaluation at the market of wild and cultured plaice captured from cold and warm water temperatures and their rigor-mortis progress.

Season	Fish	Conditions for transportation	Sample no.	Total length (cm)	Body weight (kg)	Rigor index (%)	Evaluation at the market	Market price (JPY/kg)
October (warm)	Wild	10°C, 17 h	1	45		23	Partly in rigor	3973
			2	39		23	Partly in rigor	
			3	41		21	Partly in rigor	
		0°C, 17 h	6	45		82	In rigor	3275
			7	39		72	In rigor	
			8	45		82	In rigor	
February (cold)	Wild	10°C, 10 h	7	44	1.94	0	Pre-rigor	5483
			8	42	1.31	0	Pre-rigor	
		10°C, 17 h	3	43	1.45	0	Pre-rigor	3333
			11	43	1.65	15	Almost pre-rigor	
			12	45	2.00	6	Almost pre-rigor	
		0°C, 17 h	4	42	1.29	30	In rigor	2647
			5	39	1.18	42	In rigor	
			6	40	1.22	28	In rigor	
	Cultured	10°C, 10 h	23	41	1.39	6	In rigor half toward tail	
			24	41	1.14	0	Partly in rigor	
		10°C, 17 h	21	45	2.01	54	In rigor	
			22	38	1.04	18	In rigor	

• Notes: Fish were transported using trucks specially designed for fresh fish from Hamada City in Shimane Prefecture to the Osaka Wholesale Market in October 1987 and in February 1988. Rigor indices of 0 and 100% indicate pre- and full rigor, respectively. Modified from Tanaka et al. (1991).

the state of fish rigor was apparently reflected in the market price irrespective of species (plaice or red seabream) (Tanaka 1991) (Table 4). Thus, we examined the changes in mitochondrial ATP synthase in red seabream and plaice adapted to low and high temperatures. Our results showed that fish acclimated to about 10°C contained larger quantities of ATP synthase than fish acclimated to about 25°C, irrespective of species (Itoi et al., unpublished data) (Table 5).

Concluding remarks

THE fish examined in our experiments—carp, plaice and red seabream—undergo changes in the quantity and/or quality of mitochondria in muscle tissues in order to maintain their biological rate processes and metabolism against seasonal fluctuations in their body temperature. Thus, temperature adaptation to cold water is achieved in fish by enhancement of ATP production by mitochondrial ATP synthase. Regulation of rearing conditions of fish by, for example, temperature which may increase mitochondrial ATP synthase appears to be an effective way of retarding the progress of rigor mortis in spiked fish, thereby resulting in increased commercial value for fish consumed raw (Figure 8). Such lines of investigation are continuing in our laboratory.

Table 4. Evaluation at the market of wild red seabream spiked at the brain.

Destination and month	Conditions for transportation	Sample no.	Total length (cm)	Body weight (kg)	Rigor index (%)	Market price (JPY/kg)
From Choshi City in Chiba Prefecture to the Tokyo Wholesale Market in March 1988	10°C, 9 h 20 min	5	34	1.15	100	2000
		15	32	0.90	86	
		16	32	0.95	86	
		18	33	1.00	100	
		19	33	1.10	100	
		20	30	0.85	90	
		21	34	1.20	85	
	0°C, 9 h 20 min	11	35	1.10	100	2000
		12	34	1.10	100	
		13	33	1.05	100	
		14	34	1.10	100	
From Arita City in Wakayama Prefecture to the Osaka Wholesale Market in July 1988	10°C, 6 h	14	28	0.63	15	2589
		15	30	0.76	54	
		16	28	0.70	46	
		17	30	0.74	74	
		18	34	1.04	27	
		19	30	0.70	52	
		20	31	0.79	13	
		21	29	0.72	20	
	0°C, 6 h	3	29	0.62	76	2250
		4	29	0.68	33	
		5	30	0.72	24	

Note: Rigor indices of 0 and 100% indicate pre- and full rigor, respectively. Modified from Tanaka et al. (1991).

Table 5. Changes in the FoF1-ATPase levels of carp, plaice and red seabream following cold-temperature rearing.

Factor	Carp	Plaice	Red seabream
FoF1-ATPase per unit muscle tissue	Increase	Increase	Increase
Accumulated mRNA level of FoF1-ATPase subunits	Increase	No change	Increase
Mitochondrial DNA/nuclear DNA	No change	Increase	No change
Mitochondrial protein composition	No change	No change	No change
Specific activity of FoF1-ATPase	Increase	No change	Increase

Modified from Itoi et al. (2003) and unpublished data.

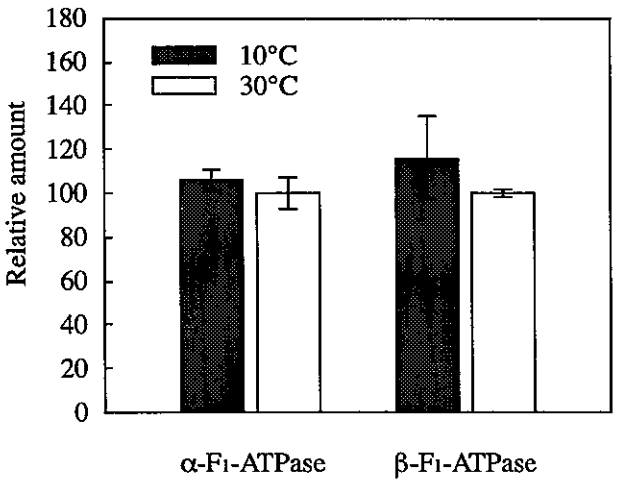

Figure 6. The contents of α- and β-F1-ATPase in mitochondria from fast skeletal muscle of carp acclimated to 10°C or 30°C. The average values (n = 3) of α- and β-F1-ATPase levels for the 30°C-acclimated carp were taken as 100. Bars represent means ± standard deviation (n = 3). Modified from Itoi et al. (2003).

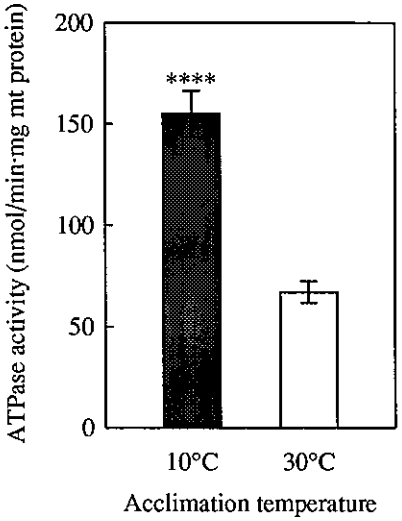

Figure 7. ATPase activity of FoF1-ATPase in fast skeletal muscle mitochondria of carp acclimated to 10°C or 30°C. Enzyme activity was assayed at 25°C. Bars represent means ± standard deviation (n = 3). Student's t-test was employed for statistical comparison (**** = P < 0.001). Modified from Itoi et al. (2003).

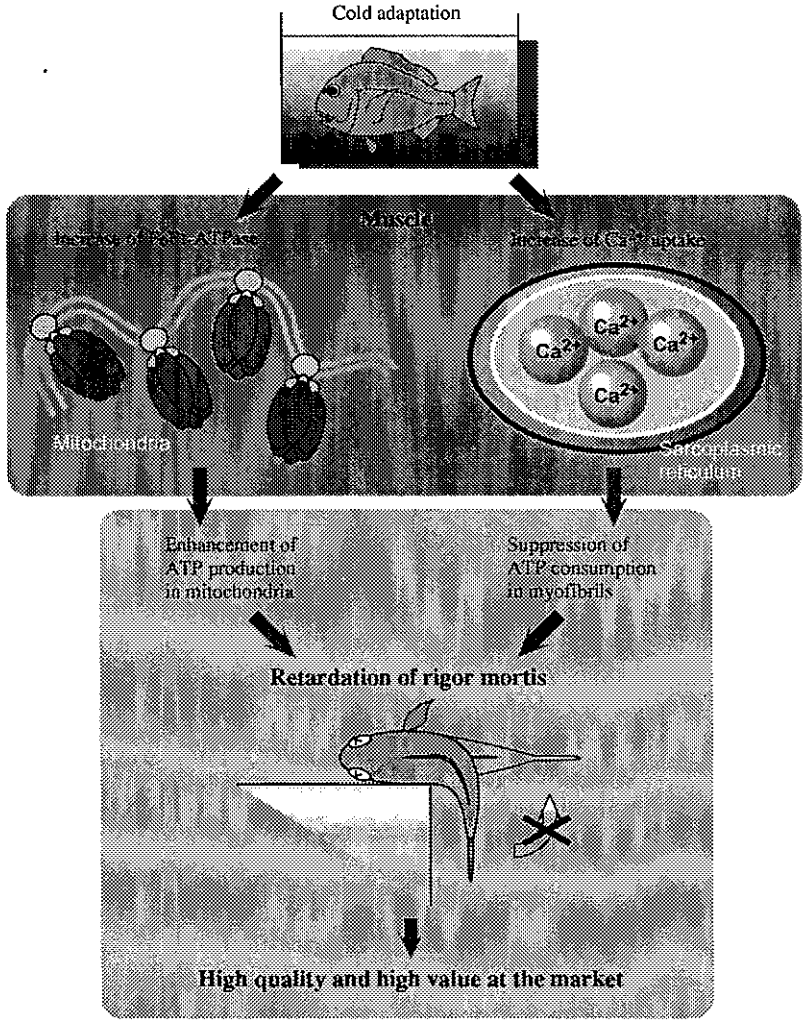

Figure 8. Enhancement of ATP production in mitochondria and extension of rigor-mortis progress in fish.

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