Zoological Studies **37**(1): 39-44 (1998)

Zoological Studies

Effect of Antifreeze Protein on Cold Tolerance in Juvenile Tilapia (*Oreochromis mossambicus* Peters) and Milkfish (*Chanos chanos* Forsskal)

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(Accepted November 2, 1997)

Su-Mei Wu, Pung-Pung Hwang, Choy-Leong Hew and Jen-Leih Wu (1998) Effect of antifreeze protein on cold tolerance in juvenile tilapia (*Oreochromis mossambicus* Peters) and milkfish (*Chanos chanos* Forsskal). *Zoological Studies* **37**(1): 39-44. The effects of administration of antifreeze protein (AFP) via anal injection, or by feeding, on the mortalities of juvenile tilapia (*Oreochromis mossambicus* Peters) and milkfish (*Chanos chanos* Forsskal) exposed to low temperature were examined.

Tilapia juveniles (2.5-3.0 g body weight) were administrated via anal injection 0 (control) or 20 μg AFP/g body weight every 2 d for 6 doses, and later were subjected to cold-tolerance test. The mortality of tilapia, 24 h after the transfer from 26 °C to 13 °C, was 53.3% in the control and 14.3% in the AFP group. Milkfish juveniles (0.9-1.1 g body weight) were given via anal injection 100 μg AFP/g body weight, 100 μg BSA/g body weight or saline every 2 d for 6 doses. After injections, milkfish were treated by gradually decreasing temperature within 4-5 d from 26 °C to 16 or 13 °C. At the end of the experiment the mortality of milkfish was 66.7%-100% in the controls (BSA and saline groups) and 13.3%-33.3% in the AFP group. In the feeding experiment artificial eel feed was given at a level of 0 (control), 100, or 1000 μg AFP/g body weight to feed tilapia juveniles (0.01-0.02 g body weight) at a rate of 20% body weight per day for 12 d. The mortality at 24 h after the transfer to 13 °C was 60% in the control, 41.9% in the 100 μg/g AFP group and 3.4% in the 1000 μg/g AFP group.

These results suggest that AFP is able to enhance the tolerance of tilapia and milkfish juveniles to exposure to low temperatures.

Key words: Antifreeze protein, Cold tolerance, Tilapia, Milkfish.

Fishes are poikilothermic animals that have been very successful in exploiting habitats with a wide range of temperatures, for example, from 35 °C (and above) in hot springs to -1.86 °C in polar oceans. In addition, habitats of many fishes encompass very considerable seasonal changes in temperature. Apart from some exceptions, fish generally maintain their mean body temperatures well within 1 degree of the ambient water temperature (Moerland 1995). Biochemical reactions of living systems are sensitive to acute changes in temperature. However, metabolic rates and activity patterns of comparable species of fish are largely conserved across a wide range of temperatures (Hazel and Prosser 1974, Crockett and Sidell 1990). The mechanisms by which fish regulate their physiological activities in extreme low temperature are the subject of active investigations (Prosser and Heath 1991, Cossins et al. 1995, Moerland 1995, Rome 1995).

DeVries and coworkers (1969) were the first to isolate an antifreeze glycoprotein (AFGP) from the blood of an Antarctic notothenid fish. AFGP has been considered to function exclusively in conferring freeze resistance by binding to ice crystals and thereby depressing blood plasma freezing points

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noncolligatively (Raymond and DeVries 1977, Rubinsky and Ikeda 1985, DeVries 1988). Since then, 4 different types of antifreeze protein, i.e., 1 AFGP type and 3 types of antifreeze proteins (AFP), have been characterized (Davies and Hew 1990). The AFP gene (type I AFP) from winter flounder (*Pleuronectes americanus* Walbaum) has been incorporated and expressed in Atlantic salmon (*Salmo salar* L.) (Shears et al. 1991). However, the low level of expression of the AFP gene was insufficient to provide any significant improvement in freeze tolerance in the transgenic salmon.

On the other hand, recent studies have indicated that AFGP or AFP facilitates the retention of normal physiological activities in the mammalian oocyte during hypothermic storage (Rubinsky et al. 1990 1991) and they protect rat livers from the impacts of hypothermic exposure (Lee et al. 1992). Hochachka (1986) has argued that in animals tolerant of low temperatures the cell membrane is less leaky than that of cold-sensitive animals. Rubinsky and his colleagues have suggested that cold-sensitive oocytes and liver cells become cold tolerant probably because AFPs reduce the leakiness of the cell membranes (Engulescu et al. 1992, Rubinsky et al. 1992). Recently, Wang et al. (1995) introduced the type III AFP gene from Newfoundland ocean pout (*Macrozoarces americanus* Schneider) into the gold fish (*Carassius auratus* Burgeri) via oocyte microinjection. Mature AFP was found in both F_1 and F_2 transgenic goldfish, and the F_2 transgenic gold fish survived better than did the control 6-12 h at 0-1 °C.

In Taiwan tremendous losses to aquaculture occur during the winter. Because of cold winds there are mass mortalities in cultured tropical fishes including milkfish and tilapia. In the present study, type I AFP purified from winter flounder was administered to juvenile tilapia and milkfish via feeding or injection, and the behavior and survival of these fishes at low temperatures were examined. The results suggest that AFP may improve cold tolerance in tilapia and milkfish juveniles.

MATERIALS AND METHODS

Fishes

Tilapia juveniles (*Oreochromis mossambicus*) obtained from the Tainan Branch of the Taiwan Fisheries Research Institute were reared in fresh water. Milkfish juveniles (*Chanos chanos*) obtained from a private hatchery were kept in 5‰ sea water. Both species were reared at 26 °C under a photoperiod of 12L:12D. Two different sizes of tilapia were used in the present study; one group was 2-3 cm in total length and 2.5-3.0 g in body weight (Experiment 1), the other group was 1-1.5 cm and 0.01-0.02 g respectively (Experiment 3). Milkfish were 3.0-3.5 cm in total length and 0.9-1.1 g in body weight (Experiment 2). Type-I antifreeze protein (AFP) was purified from winter flounder serum as described by Fourney et al. (1984) using G75 Sephadex gel filtration chromatography. AFP was dissolved in 0.9% NaCl at different concentrations for the following experiments. Experiment 1 and experiment 2 were conducted from November to January of the following year, while experiment 3 was conducted from May to June. The fish were acclimated at 26 °C over 1 mo before the experiments.

Preliminary experiment

Experiments on cold tolerance in tilapia and milkfish juveniles were conducted. All tilapia juveniles died within 30 min after direct transfer from 26 to 10 °C water. When transferred to 14 °C, juveniles showed about 33% mortality within 2 h, while juveniles survived well at 15 °C during the same period. Accordingly, direct transfer from 26 to 13 °C was used to test the cold tolerance in tilapia (Exps. 1, 3).

Milkfish did not survive after 1 h when transferred directly from 26 to 13 °C. Up to 20% of the juveniles died within 2 h at 16 °C and up to 90% during the subsequent 24 h. When the temperature was decreased gradually from 26 to 13 °C over 72-96 h, all the juveniles survived but lost their equilibrium. Survival rates varied considerably in repeated experiments. Therefore, different strategies to lower the temperature were used in Exp. 2 on milkfish.

Experiment 1

Three hundred microliters of AFP solution at dose levels of 20 μg/g (AFP/body weight) and 0 μg/g (control) was injected with blunted 27 gauge needles into the anus of tilapia juveniles every 2 d for 6 doses. The injected juveniles were transferred directly from 26 to 13 °C the day following the last injection. Mortalities were recorded 24 h after transfer. Juveniles were not fed during the experiment. A total of 10-15 juveniles were used for each test dose, and the experiments were repeated once.

Experiment 2

Milkfish juveniles were treated as described in Experiment 1 and sampled at the same time interval. AFP (100 μg/g body weight), BSA (100 μg/g body weight), and saline were administered to juveniles every 2 d for 6 doses. After injections milkfish were transferred from 26 °C to a final lower temperature by gradually lowering the temperature: Exp. 2A: 26 to 13 °C for 72 h and then 13 °C for 40 h; Exp. 2B: 26 to 14 °C for 96 h and then 14 to 13 °C for 5 h; Exp. 2C: 26 to 19 °C for 24 h and then 19 to 16 °C for 24 h. Mortalities at the end of cold acclimation in each experiment were recorded. The milkfish were not fed during the experiment. A total of 6-15 juveniles for each test dose were used for the experiments.

Experiment 3

Artificial eel feed was mixed with AFP at doses of 0 (control), 100, and 1000 μg AFP/g body weight. Tilapia juveniles were fed with the mixture at a rate of about 20% of body weight every day for 12 d. The day following the last day of feeding, juveniles were transferred directly from 26 to 13 °C water. Thereafter, the juveniles were not fed, and mortalities were recorded 24 h after transfer. Thirty individuals were used for each test dose, and 3 repeated experiments were conducted.

Because of the considerable variations in the survival rates among repeated experiments, the data from different experiments were not pooled for statistics *t*-test analysis.

RESULTS

Injection experiments

Tilapia juveniles injected via the anus with 20 μg AFP/g body weight every other day for 6 doses showed better survival at 13 °C than did the controls which were injected with saline over the same time period. Of control tilapia, 20% died within 2 h after transfer to 13 °C, and mortality was over 50% within 24 h in both Exps. 1A and 1B (Table 1). On the other hand, AFP-injected tilapia maintained a survival rate over 85% and 60% in Exps. 1A and 1B, respectively, 24 h after transfer to 13 °C (Table 1).

Although milkfish were more sensitive to low temperature than tilapia, AFP showed a similar effect in enhanceing cold tolerance in milkfish (Table 2) as in tilaphia (Table 1). In Exp. 2A, both BSA- and saline- treated groups started to show unbalanced swimming behavior within 24 h at 19 °C, however it did not occur in the AFP group until 24 h later at 16 °C. At the end of Exp. 2B (40 h at 13 °C), most of the BSA- and saline- treated groups died while nearly 80% of the AFP-treated group still survived (Table 2). Similar results were found in Exps. 2B and 2C. At the end of experiments, 66%-86% of AFP-treated groups survived, but only 0%-33% survived in BSA- and saline-treated groups (Table 2).

Feeding experiment

Similar results on cold tolerance were found in tilapia juveniles administered AFP via feeding. In Exp. 3A only 40% of the control tilapia juveniles survived 24 h after transfer to 13 °C as described above, while feeding with AFP increased the cold tolerance in tilapia and revealed a dose-related response; 60% of the 100 μg/g group and over 95% of the 1000 μg/g group survived 24 h after transfer (Table 3). Similar results were obtained in the other 2 repeated experiments, Exps. 3B and 3C, although values of mortality were different (Table 3). However, the differences between AFP groups and the respective control were not statistically significant ($p = 0.08$, *t*-test).

DISCUSSION

Both tilapia and milkfish inhabit mainly tropical and subtropical areas. Adult tilapia can survive over 12 h at either 10 or 35 °C but would die quickly at 5 °C (Trewavas 1983). Mass mortality in milkfish reared in a pond in southern Taiwan oc-

Table 1. Effect of administration of antifreeze protein (AFP) via anal injection on mortality of juvenile tilapia (*Oreochromis mossambicus*) 24 h after transfer from 26 to 13 °C

Exp. no.	AFP concentration[c]	
	0 μg/g[a]	20 μg/g
1A	53.3% (8/15)[b]	14.3% (2/14)
1B	77.8% (7/9)	40.0% (4/10)

[a] μg AFP/g body weight.

[b] Mortality and (number of deaths/total number) are indicated.

[c] No significant difference was found between control and AFP groups in both experiments ($p = 0.08$, *t*-test).

Table 2. Effect of administration of antifreeze protein (AFP) via injection on mortality of juvenile milkfish (*Chanos chanos*) transferred to low temperature[a]

	Treatment		
Exp. no.	Saline	100 μg/g[b] BSA	100 μg/g AFP[e]
2A	70.0% (7/10)[c]	100.0% (9/9)	22.2% (3/9)
2B	66.7% (5/15)	100.0% (15/15)	13.3% (2/15)
2C	83.3% (5/6)	_[d]	33.3% (2/6)

[a] The strategies of acclimation to low temperature were: Exp. 2A, 26 to 13 °C for 72 h, then 13 °C for 40 h; Exp. 2B, 26 to 14 °C for 96 h, then 14 to 13 °C for 5 h; Exp. 2C, 26 to 19 °C for 24 h, then 19 to 16 °C for 24 h.

[b] μg BSA or AFP/g body weight.

[c] Mortality and (number of deaths/total number) at the end of acclimation are indicated.

[d] No test was conducted.

[e] AFP groups were significantly different from controls ($p < 0.05$, *t*-test).

Table 3. Effect of administration of antifreeze protein (AFP) via feeding on mortality of juvenile tilapia (*Oreochromis mossambicus*) 24 h after transfer from 26 to 13 °C

	AFP concentration[d]		
Exp. no	0 μg/g[a]	100 μg/g	1000 μg/g
3A	60.0%[b]	41.9%	3.4%
3B	42.9%	_[c]	35.5%
3C	77.4%	_	53.3%

[a] μg AFP/g body weight.

[b] Thirty individuals were used for each test.

[c] No test was conducted.

[d] No significant difference was found between the control and AFP groups in both experiments ($p = 0.08$, *t*-test).

curred when temperature of the surface water decreased to near 10 °C (Ting and Chang 1974). The present study reveals that juveniles of tilapia and milkfish all die within several hours when transferred from 26 °C directly to 10 or 13 °C, respectively. The variations between the present and previous results may be due to differences in the experimental conditions, and/or different developmental stages of the fish used. As Trewavas (1983) suggested, juvenile fish prefer warmer water than do adults.

Administration of AFP, whether by anal injection or by feeding, was found to enhance cold tolerance in tilapia and milkfish juveniles. The effects of AFP in tilapia were not statistically significant, probably due to the variations in mortality among different experiments ($p = 0.08$). However, the effects in milkfish showed a significant difference between the AFP-treated and control groups when AFP was injected ($p < 0.05$). Moreover, the data of the BSA-treated group, as another negative control, provided further evidence for the specific effect of AFP on cold tolerance in these fishes. These results support previous findings that AFP can protect mammalian cells and transgenic goldfish from the impacts of hypothermic exposure (Rubinsky et al. 1990 1991, Lee et al. 1992, Wang et al. 1995).

Oral administration has been used for application of hormones or vaccinating fish in aquaculture, and it has been demonstrated that the intestinal epithelium of fishes has the ability to ingest intact macromolecular proteins (Suzuki et al. 1988, McLean and Ash 1990, Moriyama et al. 1990). However, only a small proportion of the orally delivered hormone gained access to the circulation in an immunologically and presumably biologically reactive form because most of it was hydrolyzed within the gastrointestinal tract (Moriyama et al. 1990). Rombout et al. (1985) compared the uptake and transport of intact horseradish peroxidase (HRP) in different segments of the intestine of carp (*Cyprinus carpio* L.). They found that a larger proportion of HRP absorption occurred in the 2nd intestinal segment, and they ascribed this result to the slower digestion, stronger absorptive capacity and fewer lysosomes in the 2nd segment than in the 1st segment. AFP is a small hydrophobic molecule of 38-64 amino acids, it is highly α-helical and rodlike in structure (Fourney et al. 1984). There is no information concerning digestion of AFP in fish intestine. In the case of anal injection used in the present study, AFP was thought to be

able to gain access to the posterior segment of the gut with minimum gastrointestinal digestion. This might explain why the effect of administered AFP on cold resistance in tilapia juveniles was potentially higher in when anally injected (dose: 20 μg/g) than when fed orally (dose: 100-1000 μg/g), although the effects of AFP were statistically insignificant in both tilapia experiments. The present study, agrees with previous work by Johnson and Amend (1983) who demonstrated that anal intubation resulted in better vaccination in sockeye salmon (*Oncorhyn-chus nerka* Walbaum) than either oral intubation or immersion.

Considering the difference of effects of AFP between injection and feeding experiments, another possible reason that should not be excluded is that each individual may receive various doses of AFP due to differences in amount of food taken among individuals. Those individuals which received smaller doses of AFP may show a similar tolerance to low temperature as the controls, and this may explain that the difference in survival between AFP-treated and control groups in Exps. 3B and 3C not being as evident as that in the other experiments (Exps. 1, 2, 3A).

The preservation of ion balance, despite the direct effects of temperature upon metabolism, is of critical importance to the cells of poikilothermic animals. Because temperature differentially affects the transport mechanisms involved in ion balance, changes in the body temperature of poikilothermic animals, such as fish, may disturb the normal cellular steady state (Cossins et al. 1995). The effect of hypothermic exposure upon ion regulation of cells is often considered to be due to the disparate temperature coefficients for the pump (i.e., active transport) and leakage pathways (i.e., passive fluxes); the pump generally displays greater Q_{10} (= 2-3) compared to the leaks (Q_{10} = 1-2) (Raynard and Cossins 1991). AFPs have been indicated to block calcium and potassium channels (Engulescu et al. 1992, Rubinsky et al. 1992). On the other hand, Hays and coworkers recently reported that AFGP or AFP may affect the lipid transition temperature of the membrane, i.e., preventing the liposome leakage that is induced by chilling the liposome through the transition temperature (Hays et al. 1996). It will be interesting to study the mechanisms of how AFP enhances cold resistance in tilapia and milkfish juveniles.

Transgenic research on commercially important animals was conducted initially by Palmiter et al. (1982), and this has been indicated to be one of the most powerful approaches to permanently altering the phenotype of an animal (Hew et al. 1995). A gene construct containing a complete AFP gene from ocean pout was introduced into goldfish, and a subsequent test indicated that 33% of the transgenic goldfish compared to none of the control fish survived at 0 °C for 12 h (Wang et al. 1995). To produce transgenic tilapia or milkfish with an introduced AFP gene would be a promising approach to diminish losses to aquaculture during the winter, since AFP was demonstrated to be able to enhance cold tolerance in tilapia and milkfish in the present study.

Acknowledgments: This study was partially supported by the Council of Agriculture of the Republic of China.

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抗凍蛋白對吳郭魚和虱目魚稚魚抗寒之影響

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本試驗以抗凍蛋白(antifreeze protein)經由投餵與肛門注射方式，處理吳郭魚和虱目魚稚魚，以了解它們在低溫下之抗寒效果。

吳郭魚稚魚（體重 2.5~3.0 g），經由肛門注射抗凍蛋白 0 或 20 μg/g 體重，每 2 天處理一次，共注射 6 劑之後進行低溫忍受力測試；從 26 °C 轉移到 13 °C 24 小時之後，吳郭魚稚魚之死亡率對照組是 53.3%，而處理組則為 14.3%。虱目魚稚魚(0.9~1.1 g)經由肛門注射抗凍蛋白 100 μg/g 體重，牛血清蛋白 100 μg/g 體重或生理食鹽水，每 2 天注射一劑共注射 6 劑之後，在 4~5 天之間由 26 °C 逐漸轉移到 16 或 13 °C。其結果是對照組有 66.7~100% 之死亡率（包括生理食鹽水及牛血清蛋白組），而抗凍蛋白處理組之死亡率則為 13.3~33.3%。另外在投餵實驗中，投餵鰻粉飼料含抗凍蛋白 0（對照組），100 或 1000 μg/g 予吳郭魚稚魚，投餵量是體重之 20%，共投餵 12 天；之後從 26 °C 轉移至 13 °C，對照組有 60% 死亡率，100 μg/g 抗凍蛋白組死亡率 41.9%，而 1000 μg/g 組其死亡率是 3.4%。

這些結果顯示抗凍蛋白對吳郭魚及虱目魚稚魚之低溫忍受力有增強效果。

關鍵詞：抗凍蛋白，抗寒，吳郭魚，虱目魚。

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