I. Introduction

1. The influence of climate on aquaculture fishes

Severe winter, flood, and drought have been the scourges of agriculture over the ages, bringing with them poor harvests and the threat of famine. Today, the importance of crop resistance to water stress, extremes of salinity, and harsh temperature is likely to increase further as the range of environments in which crops are cultivated expands and the incidence of extreme weather conditions increases with the specter of global warming (Shinozaki K & Yamaguchi-Shinozaki K, 1996; Zhu JK, 2001). While the importance of aquaculture as a major protein source to developing and under-developed countries is globally recognized (Tidwell JH & Allan GL, 2001), a lack of understanding of poor cold tolerance in both tropical and subtropical fish continuous to pose threat to its economical development of this industry (F.A.O., 2002).

Every year cold front sweeps across the Taiwan Strait leaving in its wake damaged aquaculture fishes, including the tilapia (*Oreochromis mossambicus*), milkfish (*Chanos chanos*), grouper (*Epinephelus coioides*) and cobia (*Rachycentron canadum*). This scenario is well known among the many major aquaculture industry sites along the densely populated coastal areas of China. The economic losses in Taiwan add up to US$10 million annually. Similarly in southern United States and Israel, water temperature during the winter may drop to levels that cause aquaculture tropical fish severe growth inhibition and mortality (Snodgrass JW,

1991).

The issue at hand is a big challenge to many farmers, agronomic officers, especially marine biotechnologist (Wise *et al.*, 2002). Accusingly, we look for an effective method to improve the cold tolerance of these culture fishes in farm. As a start, we have looked into the cold stress response of stenothermic fishes. When surrounding temperature goes below 15°C, these species lose their swimming ability and become imbalance, and finally die (Johnston IA & Temple GK, 2002). In contract, a eurythermic fish, e.g., the common carp (*Cyprinus carpio*), could survive well at temperatures as low as 10°C without showing impaired swimming ability (Watabe S, 2002). Thus, these may be physiological strategies for a fish to acclimate to low temperatures.

2. Providing assistance in energy regulation and adaptation in low temperature by creatine kinase

The swimming ability of a fish at low temperatures is fueled by muscle contraction and the efficiency of the acquisition to closely regulate the energy metabolism (Rome *et al*., 1985). Thus, we look into the most direct and important process of energy homeostasis, namely muscle and muscle-associated proteins (Bessman SP & Geiger PJ, 1981). In most muscle tissues, the ATP regeneration capacity of creatine kinase (CK, EC 2.7.3.2), the key energy metabolic enzyme (Wallimann T, 1994), is highly effective, considerably exceeding both ATP utilization as well as ATP replenishment by oxidative phosphorylation and glycolysis (Wallimann *et al*., 1992).

In our lab, we have recently cloned and characterized three muscle-specific sub-isoforms of CK from carp muscle tissue, designated M1-, M2-, and M3-CK (Sun *et al*., 1998) and have demonstrated that the M3-CK isoenzyme remains stable and maintains its enzyme activity even at lower temperatures (Sun *et al*., 2002). These results indicate that the M3-CK isoenzyme might function to allow the organization to adapt to low temperatures *in vivo*. We hypothesized that the carp M3-CK isoenzyme might substitute for the other isoforms at the lower temperature to maintain its swimming ability, enhancing its cold tolerance *in vivo* and otherwise in non-acclimated fish.

The above observation suggests a method to effectively improve

cold tolerance in tropical fish. We validate our hypothesis in a model system by introducing the heterolocus carp muscle-specific M3-CK gene into transgenic zebrafish (*Danio rerio*). The performance of this transgene was evaluated by the tolerance acquired the transgenic fish to acute water temperature changes. The physiological role of the carp M3-CK in transgenic zebrafish is evaluated by assessing its swimming performance, the behavior of balance and orientation under unfavorably low temperatures.

These results demonstrate remarkable improvement in the ability of the zebrafish to acquire cold tolerance. Therefore, it is possible to improve the survival of tropical fish in lower water temperatures by manipulating its muscle energy metabolism. In our previous work, the transgenic CMV-M3CK zebrafish shows better swimming ability and survival rates during cold shock event, but CMV promoter express constitutively, and make higher mortality in normal condition.

3. The problems which transgenic technique confronting recently

Since the generation of the first transgenic zebrafish in 1988 by microinjecting naked DNA (Stuart *et al.*, 1988), numerous successes of producing transgenic zebrafish have been reported (Amsterdam *et al.*, 1995; Bayer TA & Campos-Ortega JA, 1992; Culp *et al.*, 1991; Gibbs *et al.*, 1994; Lin *et al.*, 1994; Long *et al.*, 1997; Stuart *et al.*, 1990). Although approaches using retroviral infection (Lin *et al.*, 1994), micro projectiles (Zelenin *et al.*, 1991), and electroporation (Muller *et al.*, 1993; Powers *et al.*, 1992) have been developed to introduce transgenes into the zebrafish genome, microinjection of naked DNA is the only method that is able to produce transgenic zebrafish that express transgenes in multiple generations (Amsterdam *et al.*, 1995; Bayer TA & Campos-Ortega JA, 1992; Lin *et al.*, 1994; Long *et al.*, 1997; Noh *et al.*, 2003).

All approaches often lead to deleterious effects due to early function of the targeted gene or uncontrolled over-expression of the transgene. In many instances this leads to early lethality, precluding analysis of the developmental or physiological process under investigation as well as the generation of stable transgenic lines. To overcome this problem, several inducible gene expression systems have been developed (Delort JP & Capecchi MR, 1996; Lewandoski M, 2001; No *et al.*, 1996; Sawicki *et al.*, 1998; Yamamoto ARH & Dauer WT, 2001), one of the most promising of which is the tetracycline controlled transcription activation system (Baron U & Bujard H, 2000; Gossen M & Bujard H, 1992). Early fish transgenic research was conducted using

housekeeping gene promoters such as β-actin (Higashijima *et al.*, 1997; Hwang *et al.*, 2003; Liu *et al.*, 1990; Noh *et al.*, 2003) and elongation factor (EF) (Gao *et al.*, 1997; Kinoshita *et al.*, 2000). These promoters resulted in excess expression in various tissues.

However, tissue-specific or inducible promoters are needed to regulate foreign genes in different situations. The activities of tissue-specific promoters were assessed in zebrafish using green fluorescence protein (GFP) as a reporter gene (Udvadia AJ & Linney E, 2003). This technique has provided a powerful tool for analyzing the regulation of gene expression in living fish. The pattern of expression of GFP in these transgenic zebrafish was the same as that of the gene from which the promoter was derived. To generate transgenic aquaculture fish, tissue-specific and stress-inducible promoters from these species need to be identified. It is possible to generate an effective transgenic fish using tissue-specific and inducible promoters. Indeed, such promoters can regulate foreign gene expression faithfully. A muscle-specific inducible gene expression system would therefore provide a valuable means for addressing these and other questions about muscle physiology and function.