

## Effects of chronic cysteamine treatment on growth enhancement and insulin-like growth factor I and II mRNA levels in common carp tissues

Margaret C. L. Tse, Christopher H. K. Cheng and King Ming Chan\*

Department of Biochemistry, The Chinese University of Hong Kong, Sha Tin, New Territories, Hong Kong SAR, China

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Insulin-like growth factors (IGF) belong to a family of growth factors with structural homology to proinsulin. In our previous studies, we found that both IGF-I and IGF-II gene expression showed growth hormone (GH) dependence in the brain and liver of juvenile common carp when treated *in vivo* with GH for a short time. This present work aimed to study the effects of both the short-term and long-term GH induction of IGF gene expression using cysteamine (CSH) and fasting/re-feeding. CSH is an agent that can deplete somatostatin to increase circulating GH level. IGF mRNA levels in the flesh (muscle) and liver of common carp were determined using real-time PCR.

The chronic treatment of common carp with CSH was carried out for 63 d, with growth enhancement of the treated fish noted. Hepatic IGF-I and IGF-II mRNA levels increased in a dose-dependent manner with short-term CSH treatment, whereas IGF-I decreased and IGF-II increased in the liver after chronic CSH treatment. IGF-I and IGF-II mRNA levels in muscle were found to be elevated with the high-dose, long-term CSH treatment. Under the experimentally induced catabolic states of fasting, both hepatic IGF-I and IGF-II gene expression were significantly reduced, whereas they showed no change in muscle. After re-feeding, the hepatic expression of IGF-I in liver and muscle rebounded significantly. The hepatic IGF-II expression level showed no rebound after re-feeding, but the IGF-II level in muscle rebounded to the level of the fed group after re-feeding.

### Cysteamine: Growth rate: Real-time quantitative PCR: Gene expression

Unlike the expression of insulin-like growth factor I (IGF-I), which is highly responsive to growth hormone (GH) stimulation (Bichell *et al.* 1992; Meton *et al.* 1999), hepatic IGF-II gene expression in mammals is generally believed to be independent of GH. Meanwhile, GH may stimulate the expression of IGF-II in the extrahepatic tissues (Sara & Hall, 1990; Jones & Clemmons, 1995). However, von Horn *et al.* (2002) recently suggested that human hepatic IGF-II gene is regulated by GH.

Conflicting results also exist concerning the effects of GH on IGF-II gene expression in different fish species. In rainbow trout, GH dose-dependent responses are shown in hepatic IGF-I and IGF-II mRNA levels (Shablott *et al.* 1995), but in sea bream, only hepatic IGF-I gene expression is regulated by GH, and there is no change in hepatic and extrahepatic IGF-II mRNA levels after GH injection (Duguay *et al.* 1996). We have previously shown, in an acute *in vivo* study using ribonuclease protection assay, that both IGF-I and IGF-II exhibit GH dependence in the brain and liver of juvenile common carp (Tse *et al.* 2002). A differential expression pattern in response to the acute GH stimulation of IGF in various common carp tissues has also been studied using real-time PCR assay (Vong *et al.* 2003).

To study the effects of chronic GH treatment on IGF-I and IGF-binding proteins, different livestock species have been

used. In adult male chickens, plasma concentrations of IGF-I and IGF-binding protein 3 were markedly elevated by the continuous administration of GH. In contrast to the increases in circulating concentration of GH and IGF-I, no changes in plasma concentration of thyroid hormones, reproductive hormones, glucose or NEFA were found during chronic GH treatment (Radecki *et al.* 1997). Circulating concentrations of IGF-I were elevated by the chronic administration of GH in pigs (Chung *et al.* 1985; Etherton *et al.* 1987) and lambs (Pell *et al.* 1990).

Interactions are also found between nutritional status and IGF. The extent of nutrient deprivation determines the degree to which IGF-I synthesis and, following periods of nutrient modification, IGF-I secretion will return to normal. Food deprivation for 5 d depresses circulating IGF-I concentration, which returns to near-initial values after re-feeding (Kim *et al.* 1991; Morishita *et al.* 1993). It has also been reported that a complete return to normal IGF-I level was observed following restricted feeding (Kita *et al.* 1996). Conversely, IGF-II concentration increased following 24 h of food withdrawal in chickens (McMurtry *et al.* 1998).

The neuroendocrine regulation of GH secretion is affected by a number of stimulatory and inhibitory neurohormones

**Abbreviations:** CSH, cysteamine hydrochloride;  $\Delta C_T$ , difference between the threshold cycles for target and reference; GH, growth hormone; HSI%, hepato-somatic index; IGF, insulin-like growth factor; MLI, muscle weight divided by length index; SRIF, somatostatin.

\*Corresponding author: Dr K. M. Chan, fax +852 26035123, email kingchan@cuhk.edu.hk

acting on the pituitary somatotrophs. Somatostatin (SRIF) is the major inhibitory peptide regulating GH secretion. A large number of SRIF agonists have been synthesised (Cai *et al.* 1986), and some SRIF antagonists have recently been reported (Bass *et al.* 1996; Wilkinson *et al.* 1996), but there is a lack of biological activity of SRIF antagonists *in vivo* (Baumbach *et al.* 1998).

Cysteamine, or cysteamine hydrochloride (CSH), is an agent that works as a specific inhibitor of SRIF to increase circulating GH level and accelerate growth. Effects of CSH on GH release and growth induction have been demonstrated in sheep (McLeod *et al.* 1995*a,b*) and grass carp (Xiao & Lin, 2003*a,b*). Little is known about long-term continuous GH induction on the effect of IGF-I and IGF-II gene expression in fish. Therefore, by treating juvenile common carp with CSH for 63 d, the chronic effect of GH induction on the growth rate, and the gene expression levels of IGF-I and IGF-II were investigated in carp tissues. To our knowledge, reports of the effects of CSH on growth are scarce with regard to either fish or other vertebrates. This is also the first report linking CSH to IGF-I and IGF-II gene expression. The present study is the first report on the effects of long-term CSH treatment on mRNA levels for IGF in animals.

## Materials and methods

### Animal and experimental design

Three hundred 3-month-old (weight 2–4 g) common carp (*Cyprinus carpio*) were kindly provided by the Agriculture, Fisheries and Conservation Department of the Hong Kong Government. Carp were acclimatised at a constant temperature ( $20 \pm 2^\circ\text{C}$ ) with a controlled natural photoperiod of 16 h light–8 h dark in an aquarium room with a closed recirculation system for 1 week.

In the CSH treatment study, carp (fifty per group) with an average body weight of  $2.5 \pm 0.2$  g were randomly distributed into four replicated tanks and randomly assigned to the following groups: control (without CSH treatment), dose 1 (1000 mg/kg CSH), dose 2 (2000 mg/kg CSH) and dose 3 (3000 mg/kg CSH) treatments. After 2 d starvation, they were fed daily for 63 d with 3% of their body weight of formulated or unformulated pellets containing an active ingredient CSH with a similar outlook. The percentage of food fed (3% of body weight) was decided by feeding the carp with different amount of food to apparent satiation; all the food would be consumed within 15 min. All feeding pellets, whether or not mixed with CSH, were provided by a commercial source from Walcom Bio-chemicals Industrial Limited (Hong Kong). The mortality of the fish was less than 5% throughout the experiment.

For the fasting and re-feeding study, fifty carp with average body weight of  $3.5 \pm 0.5$  g were fasted for 31 d and subsequently re-fed with control or dose 3 pellets. The biomass (total body weights of the treatment groups) and fork lengths of the fish were monitored. Seven juvenile common carp from each treatment group were anaesthetised and then rapidly dissected, their flesh (muscle) and liver weights being determined. Tissues were wrapped in marked tinfoil or put into marked Falcon tubes (Becton Dickinson Labware, Franklin Lakes, NJ, USA), immediately immersed in liquid N and then stored at  $-80^\circ\text{C}$  for subsequent use.

### Total RNA extraction

The tissues of juvenile carp were homogenised with a Polytron PT3100 (Kinematica, Littau, Switzerland). The probe of the Polytron PT3100 was cleaned with diethylpyrocarbonate-treated water and chloroform before use. Tissue masses ranging from 0.1 to 0.5 g were homogenised with TriPure Reagent (Roche, Mannheim, Germany) solution in a ratio of 100 mg to 1 ml TriPure Reagent, and the homogenate was divided into 1 ml aliquots. Chloroform (0.2 ml) was added, and the solution was mixed vigorously for 15–30 s. The homogenate was placed on ice for 2 min and then centrifuged at  $12\,000g$  for 15 min at  $4^\circ\text{C}$ . An equal volume of isopropanol was added into the aqueous supernatant, and this was then centrifuged at  $12\,000g$  for 10 min at  $4^\circ\text{C}$ . The RNA pellet was washed with 1 ml 75%  $\text{C}_2\text{H}_6\text{O}$  and then centrifuged at  $12\,000g$  for 10 min at  $4^\circ\text{C}$ . The RNA pellet was speed-vacced for 10 min at  $65^\circ\text{C}$ , resuspended in diethylpyrocarbonate-treated water, spectrophotometrically quantified and then inspected on a 1% formaldehyde gel to check for integrity (Ausubel, 1999). The gel was then photographed under UV transillumination.

### First-strand cDNA synthesis

Total RNA (3  $\mu\text{g}$ ) was added to a nuclease-free microcentrifuge tube with 100 ng random hexamer, heated at  $65^\circ\text{C}$  for 10 min and quick-chilled on ice. The content of the tube was collected by brief centrifugation, and 6  $\mu\text{l}$  5X first-strand synthesis buffer, 3  $\mu\text{l}$  0.1 M-dithiothreitol, 1  $\mu\text{l}$  10 mM-dNTP, 1  $\mu\text{l}$  RNaseout inhibitor and 1  $\mu\text{l}$  MMLV (Moloney Murine Leukemia Virus) reverse transcriptase were added. The tube was incubated at  $37^\circ\text{C}$  for 90 min, followed by heat inactivation at  $65^\circ\text{C}$ . The first-strand cDNA products were stored at  $-20^\circ\text{C}$ .

### Primer design and conditions for real-time PCR

Gene specific primers were designed using the ABI Primer Express program (PE; Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instruction, based on the DNA sequences reported for IGF-I cDNA (Liang *et al.* 1996), IGF-II (Tse *et al.* 2002) and 18S rRNA (GenBank accession no. U87963). For IGF-I gene amplification, the primers used were 5'-CGCCTCGAGATGTATTGTGCAC-3' and 5'-CTGTATGCCGTTGCGCTCGT-3' with a 72 bp amplicon. For IGF-II gene amplification, the primers were 5'-ACCACCACAGGCCTCTCATC-3' and 5'-CATCGACTCTGTGCAA-AAAGGA-3' with an amplicon length of 66 bp. For 18S amplification, the primers used were 5'-AATCCCTGCCCTT-TGTACACAC-3' and 5'-TCCGAGGACCTACTAAACCA-3' with an amplicon length of 66 bp. HPLC-purified primers were used to minimise the effects of any side reaction products in an attempt to lead to more reliable DNA quantification.

The PCR was prepared by adding 12.5  $\mu\text{l}$  2X master mix (Brilliant SYBR Green QPCR master mix; Stratagene, La Jolla, USA), 2.5 pmol upstream primer, 2.5 pmol downstream primer, 0.375  $\mu\text{l}$  30 nM-reference dye and 1  $\mu\text{l}$  cDNA to a final volume of 25  $\mu\text{l}$ . Real-time PCR was performed on an ABI Prism 7700 machine (Applied Biosystems) with

denaturation at 95°C for 2 min, followed by forty cycles of 30 s each at 95°C, of 30 s at 65°C (IGF-I and 18S rRNA) 62°C (IGF-II) and of 30 s at 72°C, and finally a dissociation programme with samples first melted at 95°C for 15 s and then equilibrated at 60°C before slowly reheating with a ramping time of 19 min 59 s back to 95°C. The PCR condition was optimised with no peak in the dissociation curve plot (data not shown), indicating neither primer-dimer formation nor template contamination. Data were collected during the annealing and extension period in each cycle.

All standard curves for quantification of IGF-I, IGF-II and 18S rRNA (normalisation) genes were linear, with a linear correlation ( $R^2$ ) of about 1 (data not shown). The comparative  $C_T$  method (the difference between the threshold cycles for target and reference) was used; before using the arithmetic formula  $2^{-\Delta\Delta C_T}$  for quantification, an experiment was performed to validate the efficiency of the target amplification, the efficiency of the reference amplification being approximately equal to this (data not shown). Data were analysed using two-way ANOVA comparing the interactions of dose and time (d) as sources of variation. Means were compared using Bonferroni post-tests in Prism 4 on GraphPad (San Diego, CA, USA) software using a personal computer.

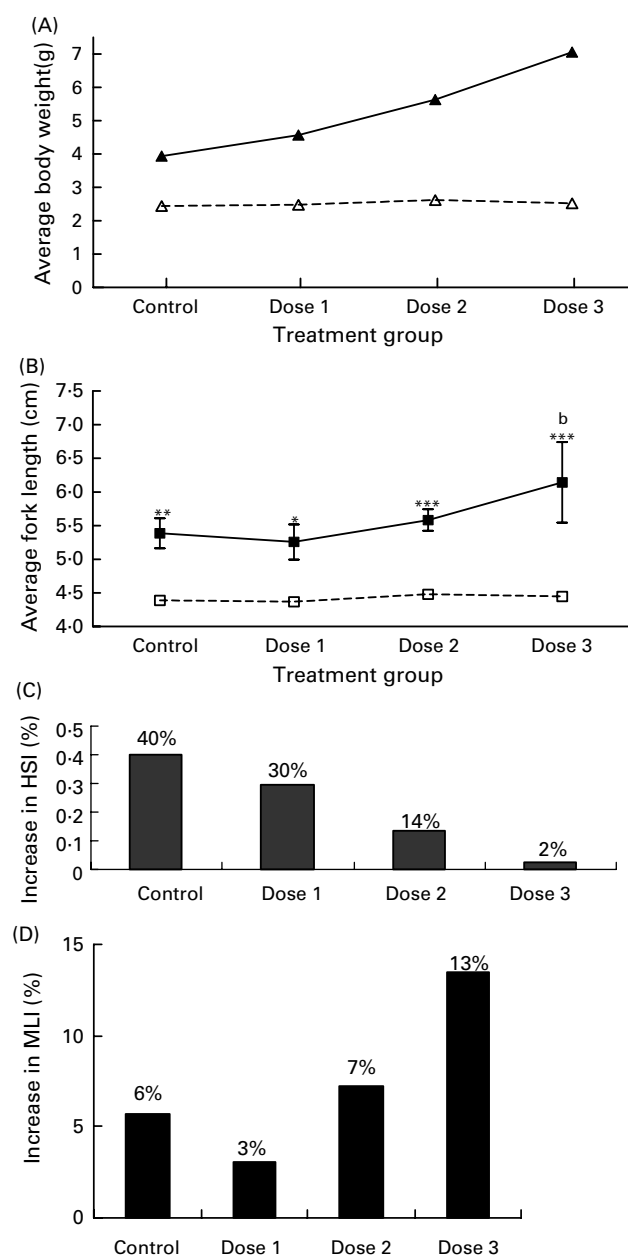
## Results

### *Body weight and fork length measurements of carp in this experiment*

Fish of a similar size were evenly distributed to different treatment groups. Growth varied between different CSH treatment groups, as indicated by the average body weight (biomass divided by number of fish; Fig. 1(A)) and fork length (Fig. 1(B)). Biomass was measured instead of individual body weight to minimise the variation in the amount of fish food taken up between individual fish within the same tank. Fish fed at higher dosages had significantly higher growth rates than fish fed at lower dosages or those not receiving CSH. The control group was the group constantly fed with unformulated pellets. The increases in biomass of the control (without CSH), dose 1 (1000 mg/kg CSH), dose 2 (2000 mg/kg CSH) and dose 3 (3000 mg/kg CSH) treatment groups between day 0 and day 63 was 61, 84, 115 and 180%, respectively; whereas the increase in fork length of the control, dose 1, dose 2 and dose 3 treatment groups from day 0 to day 63 was 22.7, 20.3, 24.6 and 38.1%, respectively.

### *Organo-somatic indices of carp following CSH administration*

The hepato-somatic index (HSI%; percentage of liver weight divided by whole body weight) and the index of muscle weight divided by length (MLI) were the indicators of fish growth in this experiment. The HSI% increased in the common carp by 40% (control), 30% (dose 1), 14% (dose 2) and 2% (dose 3), respectively (Fig. 1(C)). A dose-dependent decrease in HSI% was observed in the treatment groups compared with the control group. The increases in MLI in the common carp were 6% (control), 3% (dose 1), 7% (dose 2) and 13% (dose 3), respectively (Fig. 1(D)). In contrast to the HSI%, a dose-dependent increase in MLI was



**Fig. 1.** Effect of administration of cysteamine hydrochloride (CSH) on average body weight, fork length, hepato-somatic index (HSI%) and muscle weight divided by length index (MLI) of juvenile common carp on day 0 and day 63. (A) Effect of administration of CSH on average body weight of juvenile common carp on day 0 (Δ) and day 63 (▲). The biomass of all the fish in each tank was measured. The control group was fed constantly with unformulated pellets. Dose 1, dose 2 and dose 3 were the treatment groups, fed with 1000 mg/kg CSH, 2000 mg/kg CSH and 3000 mg/kg formulated CSH pellets, respectively. Each bar represents the pooled values for each treatment group divided by the total number of fish. (B) Effect of administration of CSH on fork length of juvenile common carp on day 0 (□) and day 63 (■). Each point represents at least six individuals for each treatment group. Mean values were significantly different from those on day 0 for the respective treatment group as measured by two-way ANOVA; \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . <sup>b</sup>Mean values were significantly different from the control and dose 3 treatment groups on day 63 as measured by two-way ANOVA. (C) Percentage increase in HSI for different treatment groups after 63 d compared with day 7 for the respective treatment groups. (D) Percentage increase in MLI for different treatment groups after 63 d compared with day 7 for the respective treatment groups.

observed in the treatment groups compared with the control group. The decrease in HSI% and the increase in MLI indicated that the body weight gain was mainly contributed by the weight gain in muscle rather than in the internal organs.

#### Validation experiments for the real-time PCR

Standard curves for relative quantification of IGF-I, IGF-II and 18S rRNA were generated, as shown in Fig. 2(A). All standard curves for the quantification of IGF-I, IGF-II and the 18S rRNA gene were linear with a linear correlation ( $R^2$ ) of about 1. The comparative  $C_T$  method was used instead of the standard curve method to eliminate the adverse effect of any dilution errors made in creating the standard curve samples.

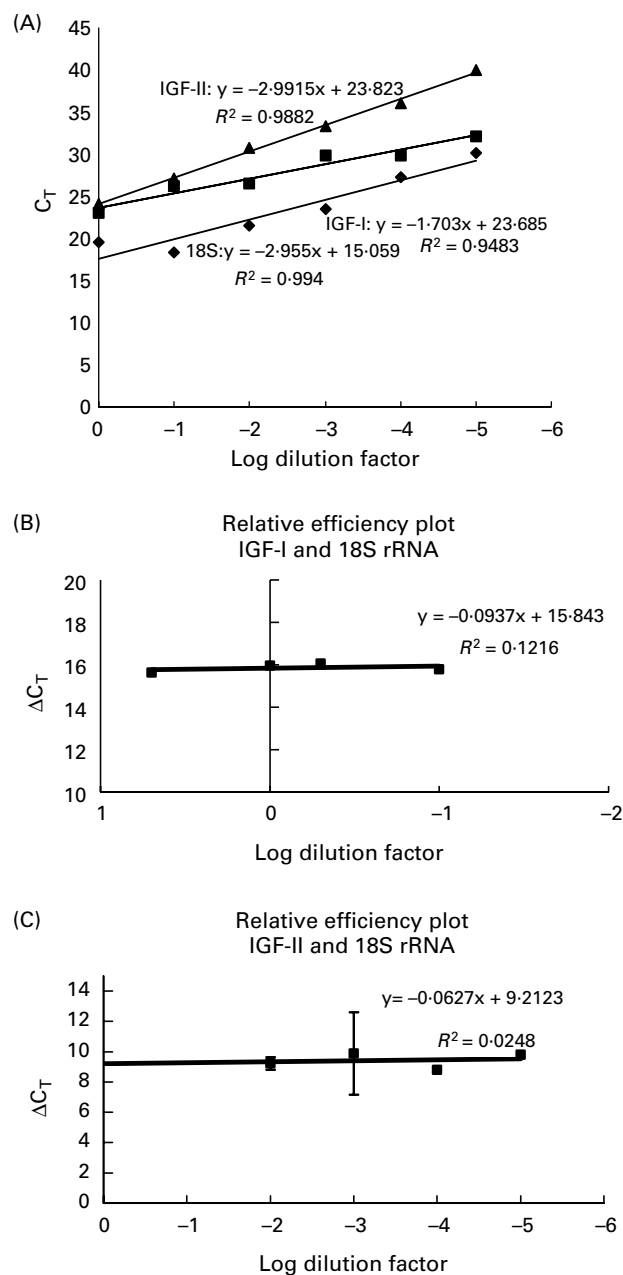
Validation experiments were performed to demonstrate that the efficiency of the target amplification and the efficiency of the reference amplification were approximately the same (Fig. 2(B,C)). We assessed whether the two amplicons had the same efficiency by looking at how  $\Delta C_T$  varied with template dilution. The slope of log dilution factor  $v$ . IGF-I (Fig. 2(B)) and IGF-II (Fig. 2(C)) was 0.0937 and 0.0627, respectively. The slope was less than 0.1, which can fulfil the criteria of using the  $\Delta\Delta C_T$  method.

The data were analysed using the arithmetic formula  $2^{-\Delta\Delta C_T}$  according to user bulletin #2 of the ABI Prism 7700 sequence detection system. Briefly, the average  $C_T$  of the target gene and housekeeping gene, respectively, were calculated. We chose 18S rRNA as the housekeeping gene because it varies least compared with other housekeeping genes such as GAPDH (Bustin, 2000) and  $\beta$ -actin (Foss *et al.* 1998). The  $\Delta C_T$  value was then determined by subtracting the average 18S rRNA  $C_T$  value from the average target  $C_T$  value. The standard deviation of the difference was calculated from the standard deviations of the target and 18S RNA values. Finally,  $\Delta\Delta C_T$  was calculated by subtracting the  $\Delta C_T$  calibrator value, and the fold-change was determined by evaluating the expression  $2^{-\Delta\Delta C_T}$ .

#### Quantification of IGF-I and IGF-II gene expression

IGF-I and IGF-II mRNA levels were determined in the liver and muscle of carp after CSH treatment using real-time PCR. As IGF-I and IGF-II mRNA may cross-react with each other due to a high degree of shared nucleotide identity of 56.4%, real-time quantitative PCR was employed in this study for its accuracy and high sensitivity. Amplification of an endogenous control, 18S rRNA, was performed to standardise the amount of sample RNA added to the reaction.

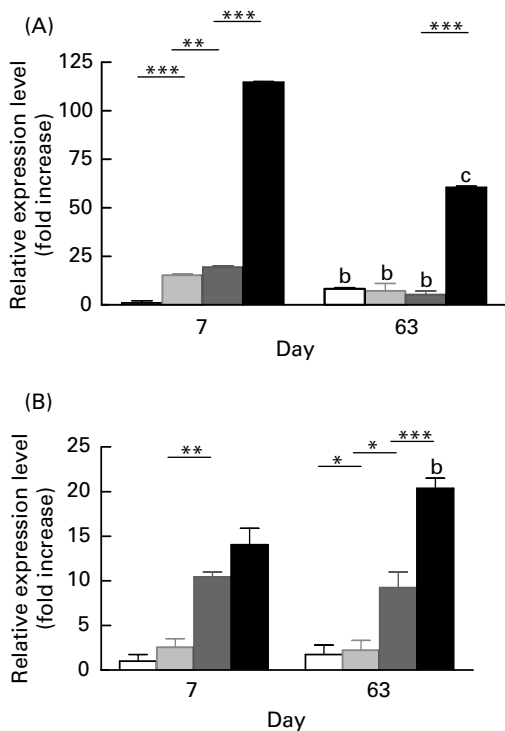
As shown in Fig. 3(A), the addition of supplementary CSH to the diet induced the expression of both hepatic IGF-I and IGF-II mRNA after 7 and 63 d of treatment in a dose-dependent manner compared with the control group (Fig. 3(A), denoted by asterisks). IGF-I gene expression was significantly increased after 63 d of feeding for the control group but significantly decreased after 63 d feeding for the dose 1, 2 and 3 treatment groups (Fig. 3(A), denoted by letters). This showed that chronic CSH treatment could suppress IGF-I gene expression in the liver. On the other hand, hepatic IGF-II gene expression showed no change after 63 d of feeding for the control, dose 1 and dose 2 treatment groups but



**Fig. 2.** Validation of the real-time quantitative PCR method. (A) Standard curves for the amplification of the insulin-like growth factor (IGF; ■) I, IGF-II (▲) and 18S rRNA (◆) target detected using the ABI Prism 7700 sequence detection system. (B,C) Validation experiments demonstrating the efficiencies of the target and reference are approximately equal. Serially diluted liver first-strand cDNA was used to perform the real-time PCR. The difference between the threshold cycles for target and reference ( $\Delta C_T$ ) between (B) IGF-I and 18S rRNA and (C) IGF-II and 18S rRNA were plotted against the log dilution factor.

was significantly increased after this time in the dose 3 treatment group, which received the highest dosage of CSH (Fig. 3(B), denoted by b).

The chronic CSH treatment induction profile in muscle was quite different from that in liver. In contrast to the situation for liver, both IGF-I and IGF-II gene expression showed no change after 63 d of feeding for the control, dose 1 and dose 2 treatment groups. For the dose 3 treatment group, however, it showed 9-fold and 4.3-fold increases in the IGF-I and IGF-II



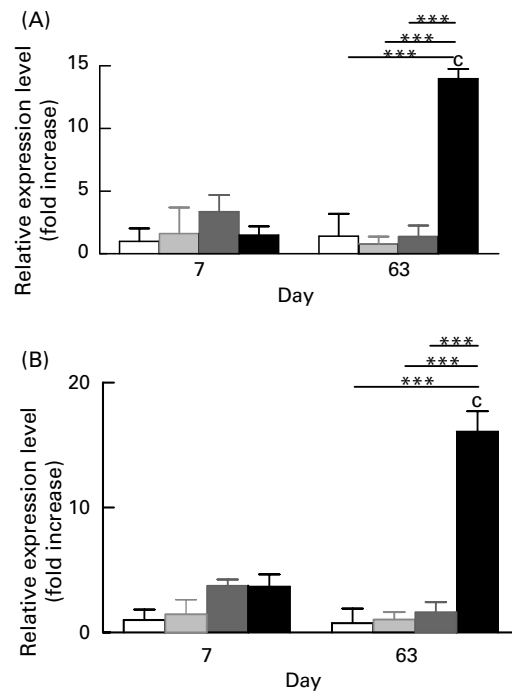
**Fig. 3.** Effect of various dosages of cysteamine hydrochloride (CSH) on day 7 and day 63 on hepatic (A) insulin-like growth factor (IGF) I and (B) IGF-II mRNA levels in carp. The control treatment group was the constantly fed group (□). Dose 1 (▤), Dose 2 (▥), and Dose 3 (▦) were the treatment groups fed with 1000 mg/kg CSH, 2000 mg/kg, CSH 3000 mg/kg formulated CSH pellets, respectively. Mean values were significantly different between different treatment groups as measured by one-way ANOVA; \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . Mean values were significantly different compared with day 7 of the respective treatment group as measured by two-way ANOVA; <sup>°</sup> $P < 0.01$ , <sup>°</sup> $P < 0.001$ .

mRNA levels (Fig. 4, denoted by c). This showed that long-term CSH treatment with the highest dosage significantly induced both IGF-I and IGF-II gene expression in muscle.

Growth decreased significantly during the fasting period (Fig. 5(A)). The dietary intake of CSH induced growth and restored growth from fasting to the extent of constant feeding by re-feeding dose 3 pellets. Hepatic IGF-I and IGF-II levels were suppressed in fasted (starved) carp (Fig. 5(B,C)). However, the level of IGF-I rebounded dramatically in the liver (210-fold) and muscle (540-fold) after re-feeding (Fig. 5(B,D)), whereas IGF-II showed no rebound in liver after re-feeding (Fig. 5(C)). IGF-II in muscle showed no response to fasting and rebounded to the level of the fed group after re-feeding (Fig. 5(E)).

**Discussion**

An improvement in fish growth performance for economic purposes can be achieved by enhancing growth rate. The growth rate of animals is modulated by their interactions with the environment, including food abundance and quality, temperature and photoperiod (Beckman *et al.* 1998). The effects of these on growth rate are in turn modulated by the endocrine system. The somatotrophic axis, which includes GH, GH receptor, IGF-I, IGF-I-receptor and IGF-binding proteins that are found

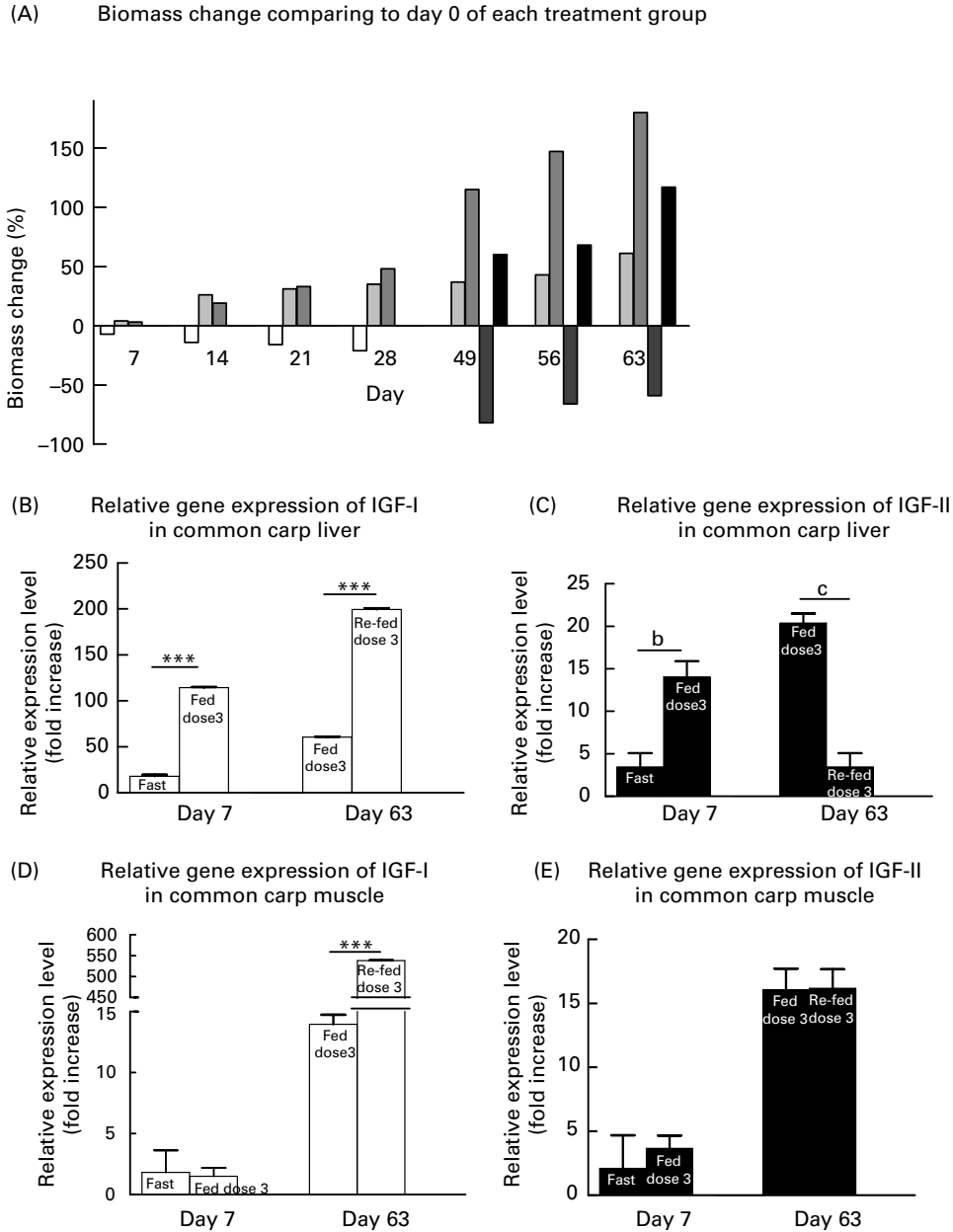


**Fig. 4.** Effect of various dosages of cysteamine hydrochloride (CSH) administration on day 7 and day 63 on muscle (A) insulin-like growth factor (IGF) I and (B) IGF-II mRNA levels in carp. The control treatment group was the constantly fed group (□). Dose 1 (▤), Dose 2 (▥), and Dose 3 (▦) were the treatment groups fed with 1000 mg/kg, CSH, 2000 mg/kg, CSH 3000 mg/kg formulated CSH pellets, respectively. Mean values were significantly different between different treatment groups as measured by one-way ANOVA; \*\*\* $P < 0.001$ . Significant difference compared with day 7 of the respective treatment group measured by two-way ANOVA; <sup>°</sup> $P < 0.001$ .

in mammals and fish (Mommssen, 1998), is believed to play a key role in the regulation of growth (Kostyo, 1999).

SRIF is the major inhibitory peptide regulating GH secretion. SRIF antagonists have recently been synthesised successfully and shown to induce GH release (Baumbach *et al.* 1998). CSH has been generally used as a tool to antagonise SRIF (Kungel *et al.* 1996). SRIF antagonists may have applications as a food additive to promote GH release, and thus enhance cultured fish growth in intensive large-scale aquaculture. They enjoy the advantage of a lack of species specificity, simple chemistry, convenient oral administration, no problems with food safety (Xiao & Lin, 2003a), minimisation of the stress introduced, such as minipumping, and a relatively low cost. Although GH and IGF-I have a more direct effect in the field of fish food enhancement to improve economic returns (Moriyama *et al.* 1990, 1993), cysteamine seems to be more applicable for farmed animals such as fish.

Carp (such as Cholympi; Hanumanthappa *et al.* 2002) are commonly used as a model to study the effect of feeding additives because of their availability and wide distribution. It has recently been proven that CSH supplementation can significantly increase serum GH level in grass carp (over 150 g; Xiao & Lin, 2003a,b). In animals, either exogenous GH administration (McLean & Donaldson, 1993) or GH transgenesis (Devlin *et al.* 1994) resulted in an increase in growth. Direct GH administration sometimes does not result in growth. Bauer *et al.* (2003) showed that the infusion of GH



**Fig. 5.** Comparison of biomass changes, insulin-like growth factor (IGF) I and IGF-II mRNA levels between fasting, constantly fed and re-fed common carp. (A) Biomass change compared with day 0 of feeding and re-feeding treatment for the control (no cysteamine hydrochloride (CSH) and dose 3 (3000 mg/kg CSH) treatment groups. The fed treatment group was the constantly fed group. For the re-fed treatment group, fish were fasted for 31 d and then fed with controls at the dose 3 level with formulated pellets from day 32 onwards. Fasting significantly decreased the biomass in both the control and dose 3 treatment groups (control fed v. control re-fed; dose 3 fed v. dose 3 re-fed; <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ ; two-way ANOVA). The percentage biomass change of the control fed group showed no significant difference compared with the dose 3 re-fed group, which indicated that the biomass returned to normal after feeding with formulated pellets. □, Fasting; ▤, Fed control; ■, Fed dose 3; ▨, Re-fed control; ▩, Re-fed dose 3. (B–E) IGF-I and IGF-II mRNA levels in (B, C) liver and (D, E) muscle of juvenile common carp after fasting and re-feeding with dose 3 CSH pellets. The fold change of gene expression was compared with that of the corresponding control treatment group. The  $P$  values were calculated by a two-tailed unpaired  $t$  test with 95% CI; \*\*\* $P < 0.001$  for IGF-I; <sup>b</sup> $P < 0.01$ , <sup>c</sup> $P < 0.001$  for IGF-II. □, control group; ▤, Dose 1; ▨, Dose 2; ■, Dose 3.

to fetal sheep suffering intrauterine growth retardation restored fetal IGF-I levels but did not improve fetal growth, further reducing fetal kidney and intestinal weights.

In the present study, we examined the effects of daily CSH treatment both on IGF-I and IGF-II mRNA levels by real-time quantitative PCR and on growth rate in juvenile common carp. Neither serum GH level (by RIA) nor GH expression level (by extraction of the pituitary) was measured because of the

small size of the juvenile fish. Nevertheless, our focus was on the regulation of growth and the GH–IGF axis by the long-term dietary intake of cysteamine. Body weight increases obviously in a dose-dependent manner (Fig. 1(A)). Although fish usually gain close to 1 g/d, restricted feeding (3% of body weight fed per day) was used in our experiments rather than feeding *ad libitum* because it allowed management to be consistent in the quantities of food delivered. This may

not have allowed the fish to demonstrate maximum growth rates, but the difference in weight gain between the different treatment groups was significant enough to demonstrate the increase in growth.

In the present study, chronic and continuous CSH treatment enhanced the growth rate in a dose-dependent manner. A dose-dependent increase in MLI and a decrease in HSI% were observed in the treatment groups compared with the control group. In muscle, an induction of IGF-I and IGF-II mRNA levels with peak orders of magnitude of 9.3 and 4.4, respectively, was obtained after 63 d of daily oral administration of CSH (3000 mg/kg). This is consistent with our previous hypothesis that IGF-II may be related to growth and development both in the early embryonic stage (Tse *et al.* 2002) and later in adults as IGF-II is expressed even more than IGF-I in most extrahepatic tissues (Vong *et al.* 2003). A recent report also supports our hypothesis by demonstrating that IGF-II mRNA levels were 3.3-fold greater in the muscle of fast-growing catfish compared with slow-growing catfish (Peterson *et al.* 2004).

It is well known that GH increases muscle growth (Machlin, 1972) and decreases fat deposition in pigs (Pursel *et al.* 1990; Wieghart *et al.* 1990), lambs (Nancarrow *et al.* 1991) and salmonids (McLean *et al.* 1994). It has also been suggested that the reduction of fat can be attributed to the direct action of GH (Etherton *et al.* 1987; Ebert *et al.* 1988). In mammals, GH is known to stimulate fat mobilisation and activate lipase to catalyse triacylglycerol hydrolysis. GH also induces absorbed nutrient repartitioning and shifts in the balance of protein deposition from fat cell to muscle cell. Smith *et al.* (1988) suggested that slower-growing genotypes of rainbow trout directed more dietary energy to fat deposition and less to protein synthesis. Later, Dunham *et al.* (2002) suggested that faster-growing transgenic common carp direct a greater proportion of their energy towards protein synthesis. It is therefore hypothesised that IGF-I and IGF-II may play a role in growth and development via their anabolic effect by stimulating GH. IGF stimulate amino acid uptake and protein synthesis in muscle and can greatly reduce the rate of protein breakdown within muscle fibres.

Nevertheless, in the present study, the induction in IGF-I and IGF-II mRNA levels was detected only for the highest dose of long-term CSH treatment. IGF-I and IGF-II mRNA levels did not differ in other treatment groups, either for the long-term or the short-term CSH treatment. This is consistent with the study carried out in catfish. The amounts of muscle IGF-I and IGF-II mRNA did not differ in recombinant GH-injected fish compared with controls (Petersen *et al.* 2005). This may suggest that an intake of 3000 mg/kg CSH exceeds the safety dosage.

In liver, a dose-dependent decrease in HSI% was observed in the treatment groups compared with the control group in the present study. This decrease in HSI% may be explained by the increase in growth rate without a mass change in the liver. The gene expression levels of IGF-I and IGF-II were also investigated in the liver as it is the main source of circulating IGF and IGF-binding proteins. It has been confirmed that acute GH treatment enhanced IGF-I gene transcription in rat liver *in vivo* (Bichell *et al.* 1992) and primary hepatocyte *in vitro* (Norstedt & Moller, 1987). Although the GH dependence of IGF-II gene expression is controversial, levels of

both growth factors in the common carp liver were significantly increased by a short time of GH treatment as assessed by both ribonuclease protection assay (Tse *et al.* 2002) and real-time PCR (Vong *et al.* 2003). The latter report showed that there was a 6-fold and a 2.7-fold increase in the levels of IGF-I and IGF-II gene expression, respectively. It was suggested that the liver is very responsive to GH stimulation to express both growth factors at a time when the common carp is rapidly growing.

Fig. 3 shows a dose-dependent increase in the abundance of hepatic IGF-I and IGF-II mRNA with CSH feeding for 7 d. This is in agreement with the supposition that IGF-I is a useful tool for estimating growth rate as IGF-I levels are positively correlated with growth rate in cattle (Breier *et al.* 1988), red deer (Suttie *et al.* 1991), sheep (Gatford *et al.* 1996), pigs (Owens *et al.* 1999; Lee *et al.* 2002), guinea pigs (Sohlstrom *et al.* 1998), gilthead seabream (Mingarro *et al.* 2002), Coho salmon (Beckman *et al.* 2004) and Chinook salmon (Beckman *et al.* 1998). The present study has provided evidence to show that hepatic IGF-II mRNA level may also serve as an additional indicator of animal somatic growth rate. In a study of catfish (Peterson *et al.* 2005), hepatic IGF-I and IGF-II mRNA was increased 4.3-fold and 14.4-fold, respectively, by day 1 in recombinant GH-injected fish compared with controls, but neither differed from controls on days 0, 2, 7, 14 and 21. This further supports the idea that CSH is a good feeding additive in terms of food stability and long-lasting effect.

With long-term CSH treatment (Fig. 3), we found that hepatic IGF-II mRNA levels increased, whereas the hepatic IGF-I mRNA level decreased significantly with peak values of about two orders of magnitude. One possible explanation for this phenomenon is a negative-feedback regulation of GH secretion resulting from the higher serum GH levels after long-term CSH feeding. There is evidence to support the fact that IGF-I suppresses the hepatic GH receptor in sheep (Min *et al.* 1996), which in turn suppresses the synthesis of IGF-I. This may explain why there is a dose-dependent increase at day 7 but a decrease at day 63, except in the control group.

On the other hand, the increase in IGF-II was maintained at a dosage of 3000 mg/kg, probably due to the fact that IGF-II is more sensitive to GH. In other words, less GH is needed to induce IGF-II even though GH receptor is suppressed. Verification by measuring the gene expression level of GH receptor in common carp is needed to support this view. Another possible explanation for the differential expression of IGF-I and IGF-II is that they may respond to GH stimulation in a different manner.

A relationship between feeding, IGF and growth in fish was also studied. Hepatic IGF-I and IGF-II mRNA levels were significantly decreased, with a shift towards catabolism during fasting. In chickens, however, food deprivation for 5 d depressed the circulating IGF-I concentration and increased the circulating IGF-II concentration after 24 h food withdrawal (McMurtry *et al.* 1998). The difference might be due to the different period of food deprivation.

Hepatic IGF-I mRNA level but not that in the extrahepatic tissues was downregulated in fasted carp. Re-fed carps exhibited a profound rebound in hepatic IGF-I level. The IGF-I expression pattern of fasted and re-fed carp is consistent

with the results of studies in chickens (Kim *et al.* 1991; Morishita *et al.* 1993) and Masu salmon (Duan & Plisetskaya, 1993). In the present study, we also investigated the IGF-I expression level in the extrahepatic tissues after re-feeding. Similar to liver, IGF-I expression level in muscle also showed a drastic increase. On the other hand, hepatic IGF-II levels showed no response to re-feeding. This might suggest that hepatic IGF-I is tightly regulated and plays an important role in the physiological consequences of stressors such as fasting and adaptation to sea water (McCormick, 1996; Mancera & McCormick, 1998). The drastic rebound in both liver and muscle showed that the regulation of IGF-I expression might not be tissue specific. On the other hand, IGF-II may possess other functions such as regulation of somatic growth, and this function may be tissue specific and more related to autocrine controls. Further study is needed into the function of IGF in fish, especially the tissue-specific function(s) of IGF-II.

In summary, we demonstrated for the first time that long-term treatment with CSH enhanced the growth of carp, suggesting that CSH would be an effective tool to enhance growth. This is also the first report linking CSH to IGF in any species. Both IGF-I and IGF-II mRNA levels in the muscle of common carp increased with long-term, high-dose CSH treatment. Hepatic IGF-I and IGF-II mRNA levels increased with CSH feeding for 7 d. However, the IGF-I mRNA level decreased and the IGF-II mRNA level increased in the liver following CSH treatment. Under the experimentally induced catabolic states of fasting, both hepatic IGF-I and IGF-II mRNA levels were reduced significantly. After re-feeding, however, liver and muscle IGF-I appeared to rebound significantly to a greater extent than was seen with the constantly fed carp. The hepatic IGF-II expression level showed no response to re-feeding. The IGF-II expression level in muscle showed no response to fasting and rebounded to the level of constantly fed group after re-feeding.

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