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Dynamics of Increase in Insulin-like Growth Factor-I mRNA Expression in Nile Tilapia, *Oreochromis niloticus*, in Response to Elevated Temperature

Emmanuel M. Vera Cruz[1,2] • Christopher L. Brown[1,3*]

[1] Department of Biological Sciences, Florida International University, North Miami, Florida 33181, USA
[2] Present address: Freshwater Aquaculture Center and College of Fisheries, Central Luzon State University, Science City of Muñoz 3120, The Philippines
[3] Present address: National Oceanic and Atmospheric Administration, National Marine Fisheries Service, The Milford Laboratory, 212 Rogers Avenue, Milford, Connecticut 06460, USA

Corresponding author: * **Christopher.L.Brownch@noaa.gov**

ABSTRACT

Insulin-like Growth Factor-I (IGF-I) is a physiological mediator and a potentially important growth indicator candidate in teleost fishes. In this study, the effects of increased temperature on the growth and hepatic IGF-I gene expression in *Oreochromis niloticus* were evaluated. Twenty all-male fish were reared separately at temperatures below 24°C for 12 days and then water temperature in 15 aquaria was gradually raised to 30°C within a day. Growth and hepatic IGF-I gene expression in five fish were obtained before the temperature change and after 2, 5 and 7 days of increasing the water temperature. The growth rate of the fish reared in the warmer temperature for 2, 5 and 7 days was significantly increased in a time dependent manner (r = 0.93). Mean hepatic IGF-I mRNA levels in fish reared at warm temperature for 2, 5 and 7 days were elevated 1.6-, 2.5-, and 3.6-fold, respectively compared to that of fish reared at cold temperature (<24°C). The IGF-I levels were significantly elevated after at least 5 days of exposure to warm temperature, which is consistent with the idea that hepatic IGF-I gene expression can be used as a short-term growth rate indicator for *O. niloticus*. A significant positive correlation was observed between days of rearing at warm temperature and hepatic IGF-I levels (r = 0.92); between specific growth rate (length) and IGF-I levels (r =0.92); and between condition factor and IGF-I levels (r = 0.55). The high positive association between IGF-I mRNA and growth rate validated the assertion that hepatic IGF-I levels are sufficiently sensitive to be used as instantaneous growth rate indicator in this species of fish.

Keywords: Growth indicator; Hepatic IGF-I; Real time qRT-PCR
Abbreviations: CF, condition factor; **DNA**, deoxyribonucleic acid; **GH**, growth hormone; **IGF-I**, Insulin-like Growth Factor-I; **mRNA**, messenger ribonucleic acid; **SGR**, specific growth rate

INTRODUCTION

Growth of fish is affected by several factors which include their genetic constitution, nutritional and environmental factors. In teleost fishes, developmental and growth processes are triggered by temperature, photoperiod and food availability. The most important environmental factor is temperature. As temperature increases, feed consumption increases to a maximum level and then decreases rapidly prior to the upper limit that the fish can tolerate (Jobling 1994). These environmental cues and internal information are processed and integrated in the brain for appropriate modification of growth through hormonally mediated pathways (Duan 1998). Central in the hormonal control of growth is the growth hormone (GH) – Insulin-like Growth Factor –I (IGF-I) axis. Pituitary GH stimulates the production of hepatic IGF-I which in turn is the primary source of circulating IGF-I (Duan 1998; Kajimura *et al.* 2001; Pierce *et al*. 2004), and which mediates the growth promoting actions of GH. Growth hormone increases the abundance of IGF-I mRNA by enhancing the transcription of the IGF-I gene as well as affecting the processing of IGF-I mRNA (Daughaday and Rotwein 1989).

Several studies have indicated that physiological and biochemical markers can be used as instantaneous indices of growth rate (Ali and Wootton 2003; Beckman *et al*. 2004; Vera Cruz *et al*. 2006; Vera Cruz and Brown 2007, 2009). Buckley *et al*. (1999) and Ali and Wootton (2003) have used of biochemical markers such as RNA: DNA ratio and lipid concentration as indices of growth rate. Circulating levels of hormones such as growth hormone (GH), thyroxine, insulin and IGF-I, on the other hand, have been proposed as growth indices in several species of fish (Pérez-Sánchez and Le Bail 1999; Shimizu *et al*. 2000; Larsen *et al*. 2001; Beckman *et al*. 2004; Vera Cruz *et al*. 2006; Vera Cruz and Brown 2007, 2009). Of these hormones, however, IGF-I is the most promising candidate in fish since this hormone acts proximally in the regulation of growth. This is evidenced by several studies indicating a significant association between IGF-I and growth rates (Uchida *et al*. 2003; Beckman *et al.* 2004; Ueda 2004; Vera Cruz *et al*. 2006). In addition, the association between IGF-I levels and growth rate is more consistent than that of GH with growth rate. Growth hormone levels can become dissociated with growth rate under some conditions, such as starvation, while the positive correlation between IGF-I and growth rate persists (Duan and Plisetskaya 1993; Duan 1997, 1998). The increase in circulating GH level during fasting is due to the significant decrease in the binding capacity of hepatic receptors to GH (Gray *et al*. 1992). This leads to reduced hepatic responsiveness to GH, in which hepatocytes become resistant to the effects of GH, thus decreasing IGF-I production in spite of high levels of GH (Thissen *et al*. 1999; Pierce *et al*. 2005a, 2005b). For these reasons, the detection

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of IGF-I is gaining more appeal as an index of growth in several species of fish.

Our previous study on *O. niloticus* indicates that three weeks exposure to warm temperature (≥ 28°C) dramatically increased the fish's hepatic IGF-I level compared to that of fasted fish and fish reared at a lower (≤ 24°C) temperature (Vera Cruz *et al*. 2006). To evaluate the possibility of using hepatic IGF-I as instantaneous or rapid growth indicator in this species of fish we evaluated the effect of increased temperature at shorter duration of exposure on the rates of growth and hepatic IGF-I mRNA expression.

MATERIALS AND METHODS

Fish

All-male Nile tilapia (*O. niloticus*) obtained from Aquasafra, Inc., Bradenton, Florida were maintained in 1700-l circular fiberglass tanks at the Marine Biology Laboratory, Florida International University (FIU). Fish were reared in continuously aerated fresh water under natural photoperiod (14 h light: 10 h dark) and were hand-fed a commercial (AquaMax) pelleted diet once a day. All fish care and experimental procedures were reviewed and approved by FIU's institutional Animal Care and Use Committee (protocol number 02-018).

Experimental procedure

Fish were randomly distributed into 20 aquaria of 64-liter volume at one fish per aquarium. Fish were fed to satiation once a day. Water temperature was maintained below 24°C for a period of 12 days (**Fig. 1**), and then the water temperature was gradually increased to 30°C within a day in 15 aquaria. The water temperature was maintained at 30 ± 1°C by a thermostatically controlled heater. During the same period, five 64-liter aquaria were kept at a water temperature of < 24°C. Standard length and body weight of 20 fish were recorded at the beginning of the experiment and prior to temperature change. Afterwards, standard length and body weight of 5 fish were recorded 2, 5 and 7 days after the water temperature changes. Two, five and seven days after exposure to warm temperature, five fish were anesthetized with tricaine methanesulfunate (MS-222) and the hepatic tissue was rapidly removed from each fish and frozen in liquid nitrogen prior to RNA extraction. During the same collection time liver samples were also collected from fish maintained at < 24°C. The fish's specific growth rate (SGR), in terms of weight and length, and condition factor (CF) were calculated as:

$$\text{SGR (weight; \%·day}^{-1}) = [(\ln W_f - \ln W_i)\cdot(t)^{-1}] \times 100$$

where W_f is the final body weight (g), W_i is the initial body weight (g) and t is the growth time (days);

$$\text{SGR (length; \%·day}^{-1}) = [(\ln L_f - \ln L_i)\cdot(t)^{-1}] \times 100$$

where L_f is the final standard length (cm), L_i is the initial standard length (cm) and t is the growth time (days);

$$CF = (BW)\cdot(L)^{-3} \times 100$$

where BW is the body weight (g) and L is the standard length (cm).

RNA isolation and cDNA synthesis

Total RNA from each liver sample was isolated using Trizol® (Invitrogen™, Carlsbad, California, USA) according to the manufacturer's protocol. Sodium chloride (Sigma-Aldrich, St. Louis, Missouri, USA) and sodium citrate (Sigma-Aldrich, St. Louis, Missouri, USA) solutions were used for precipitation of glycogen in the samples. The RNA samples were treated with a DNase I kit (DNA-free™, Ambion®, Austin, Texas, USA) in two separate reactions to remove any possible genomic DNA contamination. The total RNA was quantified using the NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, Delaware, USA), and the purity was assessed by determining the ratio of the absorbance at 260 and 280 nm. The A260/A280 values of all samples ranged from 1.9–2.0. The first strand of cDNA was synthesized using the Omniscript® reverse transcriptase kit (Promega®, Madison, Wisconsin, USA). Briefly, 1 µg of total RNA was reverse transcribed in 20-µl reaction volume [10X RT buffer, 5 µM dNTP, 10 µM oligo-dT primer plus RNase inhibitor (RNasin®, Promega®)]. The reaction was carried out for 60 min at 37°C.

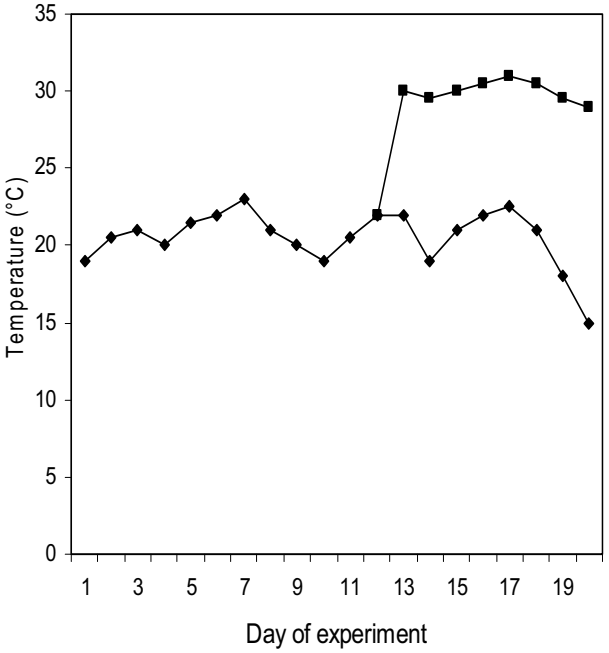


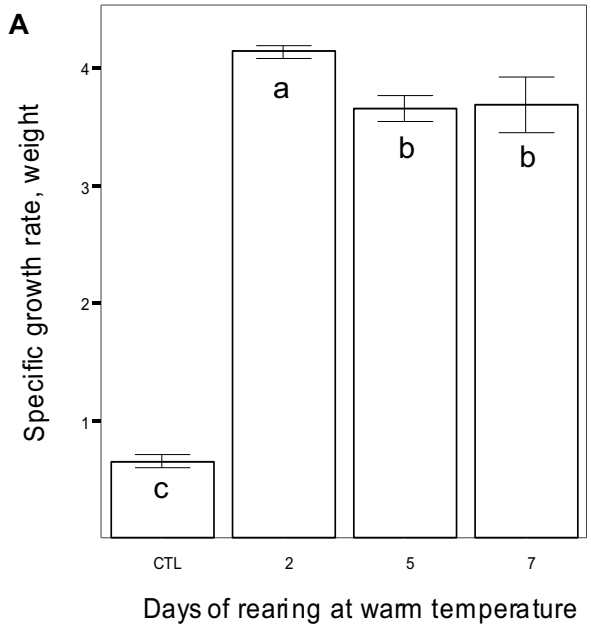
Fig. 1 Water temperature of experimental groups prior and after increasing the water temperature. (■) Warm temperature (30 ± 1°C) and (♦) Cold temperature control (< 24°C).

Quantification of IGF-1

Hepatic IGF-I levels were determined by TaqMan® quantitative real time polymerase chain reaction (PCR) as described by Vera Cruz *et al*. (2006). The real time PCR was performed on DNA Engine 2 Opticon® (MJ Research, Inc., Watertown, Massachusetts, USA), using the cycling conditions of 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min. The sequence of the probe used was 5′-TTTCAATAAACCAACAGG CTATGGCCCC-3′. The reporter and quencher dyes for the probe were 6-FAM and TAMRA, respectively. The forward primer used was 5′-GTCTGTGGAGAGCGAGGCTTT-3′, and the reverse primer was 5′-CACGTGACCGCCTTGCA-3′. Reactions for each sample were done in triplicate with each well containing 25 µl PCR mixture composed of 10 ng cDNA template, 1X TaqMan® universal PCR master mix, 900 nM forward and reverse primers and 250 nM probe. Reactions containing template but not amplified during cDNA synthesis (No Amplification Control) were used to test for any possible genomic DNA contamination in RNA preparations. Reactions without cDNA template (No Template Control) were also included to confirm that reagents were not contaminated with carryover PCR products. A serial dilution of cDNA, with the amount ranging from 0.01 to100 ng, was run to generate a standard curve (plot of the log of initial target copy number vs. threshold cycle) of IGF-1. The amount of IGF-I mRNA in each experimental sample was calculated by substituting the generated threshold cycle values to the equation derived from the standard curve (Bustin 2002). Values of IGF-I mRNA were then expressed relative to the lowest sample level measured (assigned an arbitrary value of 1).

Statistical analyses

Data were analyzed using one-way analysis of variance (ANOVA) followed by the Fisher Least Significant Difference test to determine significant ($P < 0.05$) difference between means. In the post *hoc* test, the Bonferroni corrections of the significant level were



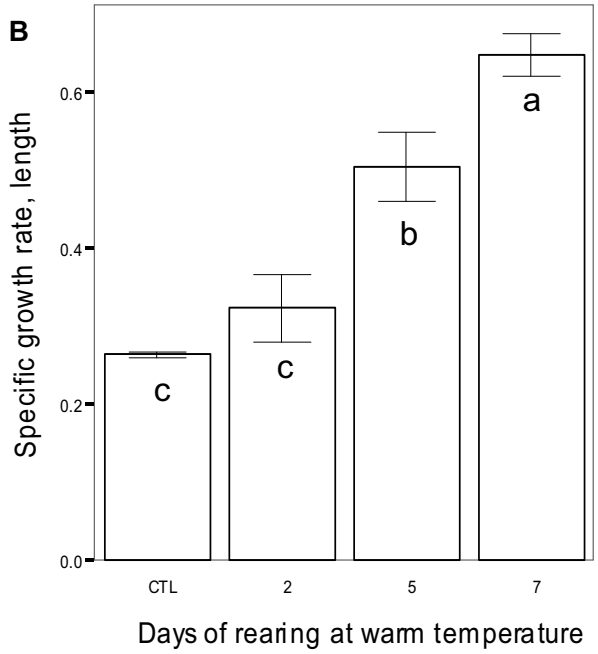


Fig. 2 Mean specific growth rates (± S.E.), in terms of (A) weight and (B) length, of the fish at the different periods of rearing at warm temperature. The control treatment (fish reared at cold temperature) is indicated by CTL. Bars labeled with different letters are significantly different ($P < 0.05$).

applied. Linear relationships of the duration of rearing at warm temperature, quantity of hepatic IGF-I mRNA and growth were assessed using linear regression and Pearson's correlation coefficient. All statistical analyses were done using version 13.0 SPSS software.

RESULTS

Temperature affects the appetite of the fish

Feed consumption of fish was observed to be influenced by temperature. The amount of feed given to fish reared at warm temperature was more than two times greater than that given to fish reared at low temperature with mean of 0.41 g·day^{-1}. It was observed that fish reared at high temperature had mean feeding rate of 1.25 g·day^{-1}, 1.28 g·day^{-1} and 1.32 g·day^{-1} for fish reared for 2, 5 and 7 days, respectively. Since feed was given slightly in excess to ensure

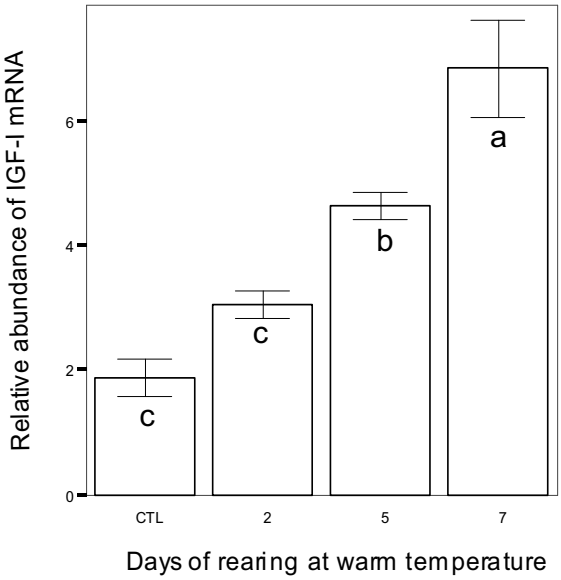


Fig. 3 Mean relative abundance of hepatic IGF-I mRNA (± S.E.) of the fish at the different periods of rearing at warm temperature. The control treatment (fish reared at cold temperature) is indicated by CTL. Bars labeled with different letters are significantly different ($P < 0.05$).

satiation feeding, it was also observed that there was greater amount of uneaten food in fish reared at lower temperature.

Temperature effects on growth rate of the fish

At the time that fish were stocked into the experimental aquaria 12 days before the change in temperature, there were no significant differences among body weights and standard lengths among the four treatments. During the 12-day period, there was also no significant difference regarding the SGR of fish in the different treatments. **Figs. 2A** and **2B** show the SGR of the fish at the different periods of rearing at warm temperature. Fish reared for two days at warm temperature (2D-W) had the highest mean SGR, in terms of weight (4.14% ± 0.05), which was significantly different ($P < 0.05$) from SGR of the other treatments. The next highest SGRs were seen in fish reared for 7 days (7D-W; 3.70% ± 0.20) and 5 days (5D-W; 3.66% ± 0.09) which were not significantly different from each other. Fish reared at cold temperature (CT) had the lowest mean SGR - weight (0.66% ± 0.05) which was significantly different ($P < 0.001$) from that of the other treatments. In terms of growth in length, 7D-W had the highest mean SGR (0.65% ± 0.02), followed by 5D-W (0.50% ± 0.04), 2D-W (0.32% ± 0.04) and CT (0.26% ± 0.002). All treatments were significantly different ($P < 0.01$) from each other except 2D-W and CT. The growth rate of fish (SGR) significantly increased with the duration of rearing at warm temperature; both in terms of weight ($r = 0.66$, $P < 0.01$) and length ($r = 0.93$, $P < 0.001$).

Temperature effects on IGF-I mRNA levels in the liver

Mean hepatic IGF-I mRNA levels in fish reared at warm temperature for 2, 5 and 7 days were elevated 1.6-, 2.5-, and 3.6-fold, respectively compared to that of fish reared at the cooler control temperature (**Fig. 3**). Statistical analysis showed that hepatic IGF-I levels were significantly elevated after at least 5 days of exposure to warm temperature ($P < 0.01$). A positive correlation was observed between days of rearing at warm temperature and hepatic IGF-I levels ($r = 0.92$, $P < 0.001$; **Fig. 4**); between SGR (length) and IGF-I levels ($r = 0.92$, $P < 0.001$; **Fig. 5**); and between CF and IGF-I levels ($r = 0.55$, $P < 0.05$; **Fig. 6**).

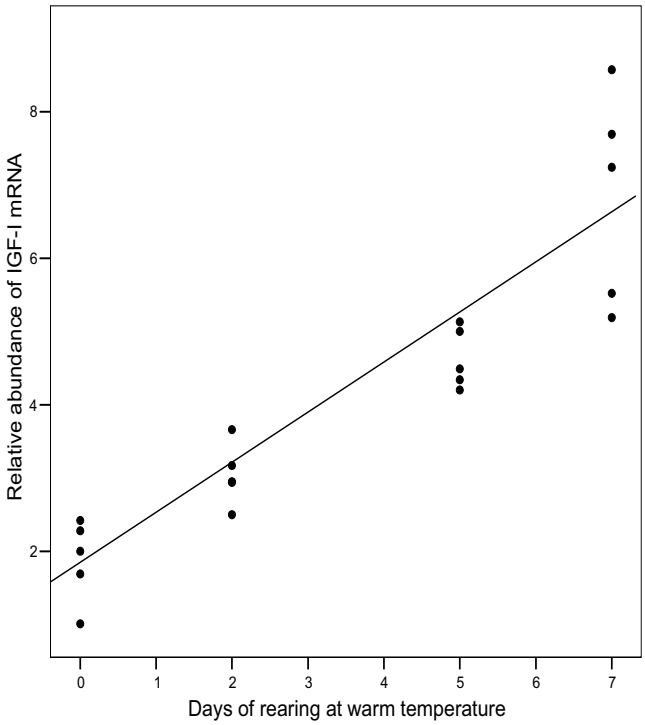


Fig. 4 Correlation of days of rearing at warm temperature with relative abundance of IGF-I mRNA. n = 20, r = 0.92 ($P < 0.001$).

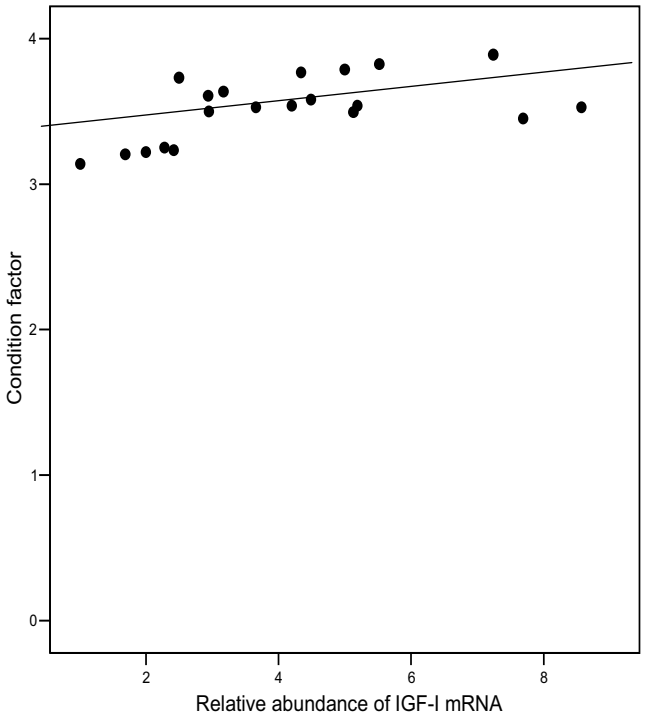


Fig. 6 Correlation of relative abundance of IGF-I mRNA with condition factor of the fish. n = 20, r = 0.55 ($P < 0.05$).

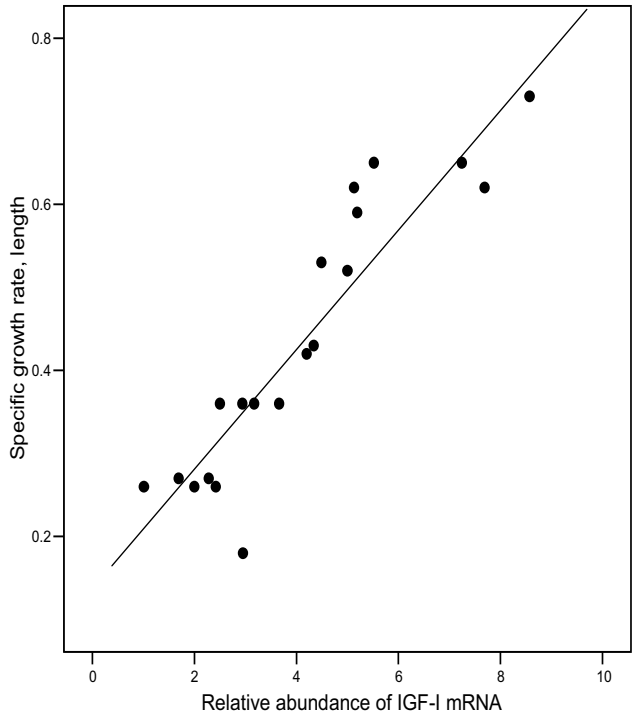


Fig. 5 Correlation of relative abundance of IGF-I mRNA with specific growth rate (length) of the fish. n = 20, r = 0.92 ($P < 0.001$).

DISCUSSION

Temperature and nutrition are the two factors most often correlated with fish growth rate. Temperature is a controlling factor that governs the rate of metabolism in poikilotherms, affecting fish appetite, digestion, nutrient absorption and consequently the rate of growth (Fry 1971). Every species of fish has its own high and low temperature tolerance for survival, growth and reproduction. Temperature tolerance range for survival is wider compared to that for growth and the range for reproduction is the narrowest. Within the optimum temperature range for growth, feed intake increases as temperature increases. This was observed in the study as fish reared at warm temperature (30 ± 1°C), which is within the optimal range for growth of 26 to 32°C, have higher food consumption than fish reared at the cooler temperature (< 24°C). The lower food intake of fish reared at < 24°C was supported by the lower feeding rate and a pattern of greater amounts of uneaten food visible in the aquaria.

The increase in appetite may be attributed to the increased metabolic rate in fish. As temperature increases, metabolic rate increases exponentially and at any given temperature, the difference between the amount of feed consumed and metabolic rate determines the energy available for growth (Jobling 1994). Since temperature influences the metabolic rate and appetite of the fish, growth of fish reared at warm temperature is believed to be a consequence of the combined effects of increased temperature and nutrition.

Water temperature or the direction of change in temperature has been found to influence environmental control of fish growth through an endocrine mechanism. In this study, increased temperature significantly elevated hepatic IGF-I levels and growth rate within 7 days and rearing in warm water produced a significant positive correlation between growth and hepatic IGF-I. In coho salmon, Beckman *et al*. (2004) similarly observed a significant positive relationship between growth and plasma IGF-I when the fish were reared in warm water but they observed disruption of associations between growth and plasma IGF-I for at least 4 weeks during a temperature decrease from 10 to 7°C. In Atlantic salmon, McCormick *et al*. (2000) observed a significant increase in plasma IGF-I within 7 days of increased photoperiod at 10°C and IGF-I remained elevated for at least 5 weeks. Similar day length but at a temperature of 2°C, however, resulted in a lower magnitude of increase in IGF-I which lasted for shorter duration. The last two studies provide evidence that low temperature limits the fish's physiological response to environmental and nutritional changes. The direct biochemical effect of lowering the temperature might alter the linear association between IGF-I and growth rate (Beckman *et al*. 2004).

Based on our data, two days exposure to elevated tem-

perature (2D-W) resulted in the highest SGR in terms of weight but 7D-W had the significantly highest SGR in terms of length. This could be explained by the transient differences in weight due to the amount of feed consumed, although this was not necessarily used for basic metabolism or growth. Since feed was given in excess, the fish in the 2D-W treatment might have consumed more food than they actually needed, with gut content changes transiently increasing the weight of fish during monitoring. This postulation is consistent with the propensity of Nile tilapia to eat excessively (Riche *et al.* 2004) and is supported by the bulgy appearance of the belly of the fish. After long periods at cold temperature, the change to ideal temperature stimulated the appetite of the fish. After five days and beyond, the fish may have adjusted to the relatively higher amount of food consumption. For these reasons, length is clearly a more definitive index of growth than weight.

Aside from the effect of temperature on fish appetite, temperature is also known to affect the endocrine system and biochemical kinetics in fish. Endocrine changes may either result from direct temperature effects on the kinetics of biochemical reactions, or in response to changing physiological requirements at different temperatures (Hochachka and Somero 2002). Temperature and nutritional status have a profound effect on circulating and hepatic IGF-I mRNA levels in tilapia (Uchida *et al*. 2003; Vera Cruz *et al*. 2006). In the present study, increased temperature brought about increased food intake and both significantly increased hepatic IGF-I mRNA levels at least 6 days after initiation of temperature change (5 days at 30 ± 1°C). This suggests that physiological signals initiating the GH/IGF-I axis response to temperature increase occur within a 6-day time period. Although not measured in the present study, the increase in hepatic IGF-I mRNA levels during exposure to increased water temperature presumably leads to an increase in circulating IGF-I levels. This was hypothesized since similar effects of temperature on IGF-I levels, but in this case in the plasma, were observed in chinook salmon (*Oncorhynchus tshawytscha*; Beckman *et al*. 1998), channel catfish (*Ictalurus punctatus*; Silverstein *et al*. 2000), and in coho salmon (*O. kisutch*; Larsen *et al*. 2001). In addition, hepatic IGF-I is the primary source of circulating IGF-I (Le Roith *et al*. 2001). A study by Pierce *et al*. (2005b) in chinook salmon supports the role of hepatic IGF-I production in the regulation of plasma IGF-I. A significant positive correlation was observed between hepatic IGF-I gene expression and plasma IGF-I levels from Day 0 to Day 22 of the experiment.

The significantly increasing growth rates of fish in terms of length (SGR; r = 0.93) over time of rearing at warm temperature may be attributed to the increasing IGF-I mRNA production in the liver during those periods. This is indicated by the observed positive correlations between hepatic IGF-I mRNA and growth, both in terms of SGR-length (r = 0.92; **Fig 5**) and CF (r = 0.55; **Fig 6**). The lesser degree of association between IGF-I level and CF compared to IGF-I and SGR-length may be attributed to the temporary effects of feed intake on weight and consequently on CF in the 2D-W fish, as compared with the 5D-W and 7D-W treatments as explained above. The high positive association between hepatic IGF-I mRNA and growth, (r = 0.92 in terms of SGR-length) shows that the hepatic IGF-I mRNA levels instantaneously indicate the growth rate status of the fish. This finding validated the assertion that hepatic IGF-I gene expression can be used as instantaneous growth rate indicator in this species of fish.

A positive correlation was observed between duration of rearing at warm temperature and hepatic IGF-I mRNA (r = 0.92; **Fig. 4**). The level of IGF-I at 7 days, however, was lower compared to the level at 21 days in our previous study (Vera Cruz *et al*. 2006) at a similar water temperature. This suggests the IGF-I mRNA level does not reach the maximum level for the set water temperature until after the 7 day period. This observation conforms with the observation of Larsen *et al*. (2001) that a rapid change in IGF-I levels observed in coho salmon following a temperature decrease was not observed in response to a temperature increase. Meton *et al*. (2000) also observed a gradual increase in hepatic IGF-I levels in refed sea bream (*Sparus aurata*).

CONCLUSION

The significant increase in hepatic IGF-I mRNA level in this study for just 6 days post-initiation of change in water temperature (5 days at 30 ± 1°C), validated the concept that hepatic IGF-I levels can be used as a short-term growth rate indicator for *O. niloticus*. The rapid and measurable change in IGF-I gene expression and the strong correlation between temperature and the quantity of IGF-I mRNA confirm its validity as an indicator. These results are in agreement with the observation by Larsen *et al*. (2001) of a significant increase in hepatic-derived plasma IGF-I in coho salmon within a week after a temperature increase from 2.5 to 10°C. The short duration involved in the significant change in IGF-I mRNA levels was also observed by Chauvigné *et al*. (2003) in rainbow trout muscle, when refeeding increased IGF-I mRNA levels by eightfold in myotomal muscle after 4 days. McCormick *et al*. (2000), on the other hand, observed elevated plasma IGF-I in Atlantic salmon within 7 days of increased photoperiod at 10°C. These studies indicate that both systemic and local IGF-I synthesis is promoted significantly for a short period by nutrition, and by environmental temperature and photoperiod.

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