Responses of growth rates and growth hormone levels of African catfish (*Clarias gariepinus*) to stocking density

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Abstract

In order to estimate responses of growth rate and growth hormone of *Clarias gariepinus* to stocking density, fish (initial weight 30.71 ± 0.89 g) were stocked in 120-L tanks at densities of 35, 65, 95, and 125 kg/m^3 for 60 days. On day 30 and 60, the fish growth rates were measured, pituitary growth hormone (GH) mRNA levels and serum GH concentrations were examined by real-time qPCR and ELISA technique, respectively. From day 0 to day 30, there were significant decreases in specific growth rates and relative weight gains at higher densities (95 and 125 kg/m^3) compared to that at lower densities (35 and 65 kg/m^3), whereas no significant effects of stocking density on the two indexes during the day 30−60 were detected. On day 30, GH mRNA levels were unaffected by stocking density, while on day 60, GH mRNA levels were significantly lower at the density of 125 kg/m^3 than in the two lower densities. Serum GH concentration increased with decreasing stocking densities but only significantly between 35 kg/m^3 and the other treatments on day 30. However, there were no significant differences between two lower densities as well as between 65 kg/m^3 and 95 kg/m^3 treatments on day 60. These results implied that effect of stocking density on SGR and RWG of *C. gariepinus* weren't uniform in different growth periods and a weak positive correlation was observed between growth rate and GH in *C. gariepinus* in the present study.

Keywords: *Clarias gariepinus*, Stocking density, Specific growth rate, Relative weight gain, Growth hormone, Real-time qPCR, ELISA

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Introduction

Somatic growth of teleost is mainly controlled by the growth axis, namely the growth hormone (GH)/insulin-like growth factor I (IGF-I) axis. GH, a single-chain polypeptide produced and secreted by somatotroph cells of the pituitary, located in the central position of the growth axis and plays a key role in enhancing somatic growth (Revol *et al*., 2005; Li *et al*., 2010). Therefore, the GH has been used as one of the primary biomarkers to evaluate somatic growth and in seeking endogenous factors affecting growth rate of fish (Ma *et al*., 2006). Some previous studies have proven that GH mRNA and protein levels were affected by fish sex (Ma *et al*., 2006; Lynn *et al*., 2009; Deng *et al*., 2014), nutrition (Gómez-Requeni *et al*., 2012) and environmental conditions (Figueroa *et al*., 2005; Lynn *et al*., 2009) and some feed ingredients (Zhou *et al*., 2013).

Stocking density is one of the major factors in aquaculture influencing growth, and the adverse and null effects on growth performance at high stocking densities depend on the fish species and their age (Salas-Leiton *et al*., 2010). African catfish (*Clarias gariepinus*) is an important commercial species in Africa, Europe and Asia owing to its fast growth, high stocking-density capacities and high resistance to poor water quality and aquatic hypoxia. The fish is normally cultured in whedos, as well as in intensive recirculation systems (Volckaert *et al*., 1999; Toko *et al*., 2007; Van de Nieuwegiessen *et al*., 2009). Some investigations have been done to study how stocking density influences the growth of African catfish reared in whedos (Toko *et al*., 2007) and the growth and welfare in recirculation systems (Van de Nieuwegiessen *et al*., 2008 and 2009). Highly intensive concrete pond systems with daily 150% water exchange, for rearing *C. gariepinus*, have been successfully developed in Tianjin Deren aquaculture centre, China. A study mimicking the practical experiment was carried out to detect the effects of stocking density on *C. gariepinus* daily weight gain, feed conversion ratio, as well as welfare parameters have been reported (Dai *et al*., 2011; Wang *et al*., 2013). Nevertheless, no information about the GH responses promoted by stocking density is available in *C. gariepinus*. For these reasons, herein, we investigated how stocking densities affected specific growth rate and relative weight gain, and measured the variation of the GH mRNA levels in the pituitary and protein levels in the serum of *C. gariepinus* reared at four stocking

densities. Moreover, we assessed the relationship between growth rates and GH (GH mRNA level and serum GH concentration).

Materials and methods

Experimental design and handling

Fish and the rearing conditions used in this study were the same as the study reported by Dai *et al.* (2011) and Wang *et al.* (2013). In brief, after 2 weeks acclimation to laboratory conditions, *C. gariepinus* juveniles (mean weight 30.71±0.89 g) were randomly divided into four density-stocked treatments (35, 65, 95 and 125 kg/m^3) with 3 replicates per treatment. The fish were reared for 60 days in plastic tanks containing 120 L of water continuously aerated by air compressor. The water temperature ranged from 26 to 28°C. Fish were hand-fed commercial catfish floating pellets at 2.0% of body weight twice a day (08:00 h and 16:00 h) during the trial. To mimic the practical water exchange, the 75% of water volume was renewed with aerated water (27±1°C) 2 hours after each feeding. Fish were fasted 24 h before each sampling.

Growth performance

Fish were carefully counted and weighed on day 0, 30 and 60 after the commencement of the assay. Fish mean body weight (MBW) was calculated using the sum of fish weight divided by the fish number in each tank. Growth performances were expressed as the mean body weight, specific growth rate (SGR) and relative weight gain (RWG). SGR and RWG were calculated according to the following formulae (Varela *et al*., 2010):

$$SGR\ (\%/d) = (\ln W_f - \ln W_i) / t \times 100$$

$$RWG\ (\%) = 100 \times (W_f - W_i) / W_i$$

Where, W_f and W_i are fish mean weight (g) at the end and at the beginning of each analyzed period, respectively; t is the total number of days of this period.

Sampling procedures

To perform GH gene expression in pituitary analysis and GH protein in serum determinations, on day 30 and day 60, between 08:00 and 09:00 h, randomly selected fish per tank were carefully removed by dip net, quickly anesthetized by immersion in water with 200 mg/L MS-222 and blood was withdrawn from the caudal vessels in the hemal arch of the fish using a syringe. Serum was obtained according to the method of Wang *et al*. (2013). Sera from 3–5 fish were pooled in equal aliquots for each specimen and six

specimens were prepared for each tank (18 specimens per treatment) and subsequently stored at -20°C until analysis for GH concentrations. After blood withdrawal, fish were immediately killed by spinal transaction and the pituitary were excised, frozen in liquid nitrogen and stored at -80°C until total RNA extraction.

GH mRNA expression analysis by real-time qPCR

Pituitary pooled as sera specimens cited above (18 specimens per treatment) was mechanically homogenized and total RNA was isolated using Trizol reagent (Fermentas, Lithuania) according to the manufacturer's protocol. RNA samples were qualitatively analysed on 1% agarose gel and quantified on a NanoPhotometer (Implen, Germany). Total RNA (1 µg) from each specimen was reverse-transcribed using the RevertAid™ First-Strand cDNA Synthesis Kit (Fermentas, Lithuania). Real-time qPCR analyses were carried out on an iQ™ 5 real-time PCR detection system (Bio-Rad, USA). Specific primers for GH and 18S rRNA as a reference gene were designed using the software Primer 5 based on the sequences of *C. gariepinus* GH (previous cloned at our laboratory and submitted to GenBank database under accession No. FJ823972) and 18S rRNA (GenBank accession No. AJ876383), respectively. Initially, primer sets of GH and 18s rRNA, both were separately assessed, including specificity of real-time qPCR verified by melt curves as well as their efficiency monitored by standard curves which were generated by amplifying 10-fold serial dilutions of cDNA using 5 points for each gene of interest. After that, only the primer pairs (Table 1), possessing good specificity (melt curves with single peak) and efficiency (between 90% and 105%), as well as the efficiency difference between amplifying target and reference gene less than 5%, were employed to analyse the GH mRNA expression in pituitary of *C. gariepinus* reared at different stocking densities. PCR reactions including primer sets assessment and specimens analysis were done in a final volume of 20 µL using SsoFast™ EvaGreen® Supermix With Low ROX following the instruction manual of the kit (Bio-Rad, USA). The cycling program for both genes was: 95°C for 3 min, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s, and finally a melt-curve analysis was performed over a range of 65-95°C to ensure the qPCR specificity.

Table 1: Primer sequences used for real-time qPCR assays.

Gene Name	Primer		Amplicon size (bp)
	Name	sequence	
Growth hormone (GH)	GH-F	5′−GAACCTGGGCAACCCTAAC−3′	195
	GH-R	5′−AAGCAAGACAGCAGACGGA−3′	
18S rRNA	18S-F	5′−GCCGTCAAACTCGCCTGAATACCT−3′	176
	18S-R	5′−AAATGCTTTCGCTTTCGTCCGTCT−3′	

Each qPCR reaction was performed in duplicate and the duplicate sample Ct values with a standard deviation (SD)>0.5 were rerun. Amplification of GH was run in parallel with the reference gene. GH mRNA relative expression levels in pituitary were calculated according to: $2^{-\Delta Ct}$, ΔCt=Ct (GH) − Ct (18s rRNA), (Chen *et al*., 2012).

Serum GH concentration analysis

The serum GH levels (18 specimens per treatment) were measured using enzyme-linked immunosorbent assay (ELISA) according to the method (Drennon *et al*., 2003; Li *et al*., 2010) with some modifications. Sangon Biotech (Shanghai, China) Co., Ltd prepared the polyclonal antisera against *C. gariepinus* GH in two New Zealand raised rabbits using recombinant *C. gariepinus* GH protein via purification and renaturation as an antigen (which was also used as standard in ELISA analysis). The antisera titre to the antigen was above 1:250000. The antisera were stored in aliquots at -20°C and subsequently, the thaw antisera were directly used at appropriate dilutions for ELISA assay. Prior to ELISA assay, coating buffer, blocking solution as well as the concentration of coating antigen and antisera were optimized using checkerboard titration. Assay procedures of our ELISA were as follows. 96-wells microtiter plates were coated with 100 µL per well of 200 ng/mL of antigen in carbonate buffer (0.015 M Na_2CO_3 and 0.035 M $NaHCO_3$, pH 9.6) and incubated overnight at 4 °C. The plates were washed three times with PBST (0.005 M Na_2HPO_4, 0.14 M NaCl, 0.0015 M KH_2PO_4, 0.0027 M KCl, and 0.05% Tween 20, pH 7.4) using an automated microplate washer (Immunowash 1575, Bio-Rad, USA), and then blocked with 200 µL per well of 3% ABS-PBST for 2 h at 37°C. Following another washing step, 50 µL per well of standard dilutions (serially diluted 1:2 with 1%ABS-PBST and the concentration ranged from 100 ng/mL to 0.78 ng/mL)

or the diluted serum samples (diluted 1:1 with 1% ABS-PBST) and 50 μL per well of antisera (diluted 1:8000 with 1% ABS-PBST) were added and incubated for 2 h at 37 °C. The plates were washed three times again and 100 μL per well of goat-anti-rabbit Ig G-HRP (Sangon Biotech Co., Ltd, Shanghai, China) diluted 1:4000 in PBST were added and incubated at 37°C for 1 h. Following another washing step, 100 μL TMB (tetramethyl benzidine) substrate solutions were added to each well and developed in the dark at room temperature for 15 min. After the colour development, the reactions were stopped by adding 50 μL of 2 M sulfuric acid and the absorbance values of each well were measured at 450 nm using a microplate reader (Synergy™ 2, BioTek, USA) and recorded using a computer. All ELISA experiments were conducted in triplicate. Standard curve was generated by plotting absorbance against the logarithm of analyte concentration (García-Nieto *et al*., 2010). The concentrations of GH in serum specimens could be known as ng/mL based on the standard curve.

Statistical analysis

Data in this study were expressed as means±standard error (SE) and subjected to one-way analysis of variance analysis. Post-hoc multiple comparisons among means between treatments were made using Duncan test, with the differences considered to be statistically significant at $p<0.05$. All statistical analyses were performed using the software SPSS version 11.5 for Windows.

Results

Mean values of growth parameters, including of MBW, SGR and RWG of *C. gariepinus* juveniles reared at four different stocking densities of 35, 65, 95 and 125 kg/m^3 are given in Table 2. During the growth range of 30.71±0.257−59.58±1.21 g (day 0−30), significant decreases in SGR and RWG at higher densities (95 and 125 kg/m^3) compared to lower densities (35 and 65 kg/m^3) ($p<0.05$) were observed and there were no significant differences between the 35 and 65 kg/m^3 treatment as well as between the 95 and 125 kg/m^3 treatment ($p>0.05$); during growth range of 59.58±1.21−92.79±2.17 g (day 30−60), decreasing SGR and RWG with increasing stocking density were detected but the results did not show significant difference ($p>0.05$) between any two treatments.

Table 2: Mean values (±SE) of Growth rates of *Clarias gariepinus* reared at different stocking densities over a 60-day experimental period.

Parameter	Day	Stocking density (kg/m^3)			
		35	**65**	**95**	**125**
	0	31.01±0.27	29.77±0.19	30.82±0.68	31.22±0.52
MBW(g)	30	64.75±0.70 ^{a}	60.79±0.25^{ab}	57.40±1.96^{bc}	55.38±1.63^{c}
	60	100.94±1.97^{a}	96.64±2.22^{a}	90.70±0.69^{b}	82.87±1.56^{c}
	0-30	2.45±0.05^{a}	2.38±0.01^{a}	2.07±0.05^{b}	1.91±0.15^{b}
SGR(%/d)	30-60	1.48±0.03	1.54±0.09	1.53±0.13	1.35±0.16
	0-60	1.97±0.04^{a}	1.96±0.05^{a}	1.80±0.04^{b}	1.63±0.01^{c}
	0-30	108.83±3.09^{a}	104.21±0.68^{a}	86.16±2.97^{b}	77.6 7±8.17^{b}
RWG(%)	30-60	55.85±1.36	59.01±4.27	58.44±6.32	50.07±7.25
	0-60	225.54±7.20^{a}	224.75±9.49^{a}	194.60±7.41^{b}	165.45±0.61 ^{c}

MBW=mean body weight; SGR=specific growth rate; RWG=relative weight gain. Values within a row with different superscripts differ significantly (p<0.05).

However, throughout the trial period (day 0−60), similar results to the trial period of day 0−30 were found, except that there were significant decreases (p<0.05) in SGR and RWG in the 125 kg/m^3 treatment compared to the 95 kg/m^3 treatment.

As shown in Fig. 1, the GH mRNA relative expression levels on day 30 were not affected by stocking density (p>0.05), while on day 60, it decreased with increasing stocking density and significantly decreased in the 125 kg/m^3 treatment compared to that in the 35 and 65 kg/m^3 treatments (p<0.05).

In addition, on day 30, serum GH concentration was down slightly when stocking density was up, but it was significantly higher at stocking density of 35 kg/m^3 compared to that in the rest of the densities (p<0.05). On day 60, serum GH concentration decreased obviously with increasing stocking densities but the results didn't show significant differences between the 35 and 65 kg/m^3 treatments as well as between the 65 and 95 kg/m^3 treatments (Table 3).

Discussion

In Tianjin Deren aquaculture centre, China, *C. gariepinus* are farmed in highly intensive concrete pond systems with about 150 % daily water exchanges. There are two stages in the fish farming. In first farm stage, approximately average 30 g juveniles grow to an average size of 80−100 g, thereafter the fish are graded and reared to market size.

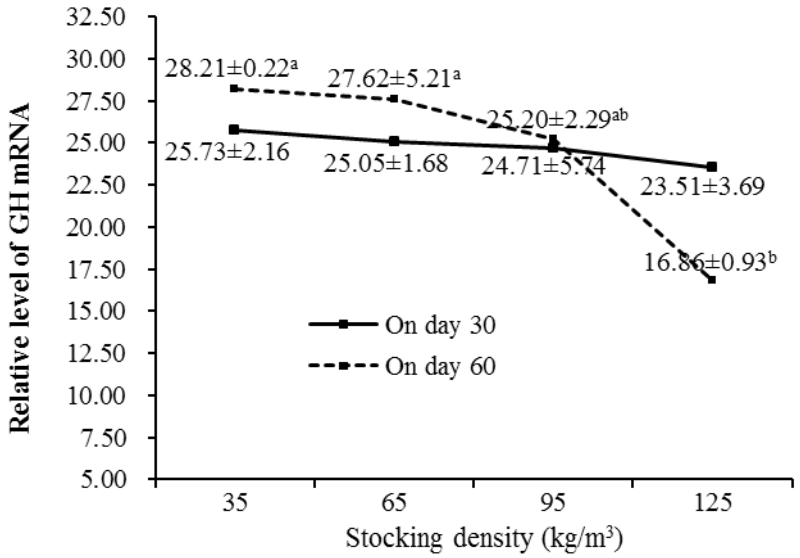

Figure 1: Changes in the relative expression levels of GH mRNA in the pituitary of *Clarias gariepinus* juveniles reared at different stocking densities.

Values with different superscripts on the same lines are significantly different (p<0. 05).

Table 3: Mean values (±SE) of GH concentrations (ng/mL) in serum of *Clarias gariepinus* reared at different stocking densities.

Day	Stocking density (kg/m^3)			
	35	**65**	**95**	**125**
30	3.61±0.11^a	3.32±0.09^b	3.26±0.05^b	3.23±0.07^b
60	3.73±0.10^a	3.46±0.13^{ab}	3.12±0.15^b	2.73±0.13^c

Values within a row with different superscripts differ significantly (p<0.05).

Under experimental conditions mimicking the practical farming, the effects of stocking density on daily weight gain, feed conversion ratio, haematological and biochemical indexes as well as non-specific immune and antioxidant indices of *C. gariepinus* (weight range 30–100 g) were reported by Dai *et al*. (2011) and Wang *et al*. (2103). In this study, SGR and RWG were used to assess the effects of stocking density on the growth performance of *C. gariepinus*. During day 0−30, for a growth range of 30.71±0.257−59.58±1.21 g, the higher densities (95 and 125 kg/m^3) showed significantly lower SGRs and RWGs but no influence of stocking density on SGRs and RWGs for the growth range of 59.58±1.21−92.79±2.17 g. This finding implies that the effects of stocking density on SGR and RWG of *C. gariepinus* were not uniform in different growth stages. The result is in accordance with previous studies that showed diverse effects of density on

growth of different life stages, indicating density dependent or independent, in *C. gariepinus* (Hossain *et al*., 1998; ToKo *et al*., 2007; Van de Nieuwegiessen *et al*., 2008 and 2009) and in other fish, such as *Solea senegalensis* (Sánchez *et al*., 2010). The higher stocking densities (95 and 125 kg/m^3) had an important effect on growth, leading to a significant decrease in SGR and RWG at densities of 95 and 125 kg/m^3 than the rest of densities for the 60-day experiment, which could be attributed mainly to the first 30 days of the experiment.

Previous studies have shown a significantly positive correlation between GH mRNA level and growth rate in *Cyprinus carpio* (Figueroa *et al*., 2005), *Oreochromis niloticus* (Ma *et al*., 2006), *Perca flavescens* (Lynn *et al*., 2009), *Scatophagus argus* (Deng *et al*., 2014) and so on. However, in our study, although higher densities (95 kg/m^3 and 125 kg/m^3) showed a significant influence on SGRs and RWGs during the first 30 days, the impact of stocking density on GH mRNA levels appeared to be negligible on day 30. Significantly lower SGRs and RWGs were found at densities of 95 and 125 kg/m^3 during the experimental 60 days, while, significant differences in GH mRNA levels were not observed between the density of 95 kg/m^3 and the densities of 35 as well as 65 kg/m^3 on day 60. These results mean that there was no consistent difference between GH mRNA level and growth rate, implying a weak positive correlation between the two factors in *C. gariepinus* juveniles in the present study. This is not in accordance with previous studies cited above. Complicated correlations between circulating GH and weight growth in fish have been reported in previous studies. Serum GH levels are negatively correlated with growth in *Mystus macropterus* (Wang *et al*., 1999) and *Salmo salar* (Stefansson *et al*., 1999) or positive correlation in *Oncorhynchus kisutch* (Young *et al*., 1989). An increase of plasma GH levels is accompanied by a subsequent increase in growth in *Sparus aurata* (Pérez-Sánchezl *et al*., 1994). However, taken together, our results indicated a weak positive correlation between growth rate and serum GH concentration, which is in accordance with findings on *Oreochromis nilotica* (Qiang *et al*., 2012).

In fact, in the GH/IGF-I axis, GH secreted from pituitary binds to its receptor and stimulates IGF-I synthesis and secretion from a wide variety of tissue cells, promoting somatic growth through IGF-I receptors (Duan, 1998;

Li *et al*., 2010). Hence, we can't illuminate the endogenous cause of fish growth only based on the information from a single factor (e.g. GH). Unfortunately, due to the lack of specific assays, we were not able to measure the mRNA and protein levels of IGF-I and GH receptor in *C. gariepinus* under the present experimental conditions, and further research is needed to finely investigate the potential endocrine pathways involved in *C. gariepinus* growth.

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