Pakistan J. Zool., pp 1-10, 2021. **DOI**: https://dx.doi.org/10.17582/journal.pjz/20200328130355

Insights in the Size Heterogeneity of *Lates calcarifer*: Expression of Growth and Immune-related Genes

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Article Information
Received 28 March 2020
Revised 18 May 2020
Accepted 30 May 2020
Available online 18 December 2020

Authors' Contribution
ZM and GY designed this study. ZF, RY, XW and SZ conducted the tests. ZF, ZM, GY, XT drafted the manuscript.

Key words
Size heterogeneity, *Lates calcarifer*, Gene expression, Growth, Immune

ABSTRACT

Significant size heterogeneity in barramundi *Lates calcarifer* juveniles has been frequently reported in hatchery practices. Huge variation of mortality in different size grade of juvenile fish has also been observed. In order to explore the effect of size heterogeneity on gene expression level of barramundi juveniles, we graded them according to body length and selected growth-related genes (GHRH, GH, GHR, IGF-I, IGF-II, MSTN-1 and MSTN-2), and immune-related genes (IL8, IL10, TGFβ1, TNF, INF-γ1, Mx, CRP, C3, C4, mTOR, mLST8, eIF4E, HSP70 and HSP90) to measure their relative expression level. Results from the present study indicate that the expression level of GHR, IGF-I and IGF-II is consistent with body length, but there is no similar rule between GHRH, GH, mstn-1 and mstn-2. TOR pathway related genes were more actively expressed in L grade. Heat shock protein has no obvious rule in body length grade. L grade pro-inflammatory cytokines were up-regulated, but anti-inflammatory cytokines were down-regulated and inflammatory markers were up-regulated, indicating an obvious inflammatory response. The expression levels of S and M grade anti-inflammatory and proinflammatory cytokines was more balanced. This study provides the basis for finding the relationship between size heterogeneity and gene expression level of barramundi juveniles, and may provide insights for further understanding on the causes of size heterogeneity.

INTRODUCTION

Size heterogeneity exists in many species within a batch in the growing period. This phenomenon can be caused by multiple factors including internal and external factors (Geffen, 2002). Intrinsic factors include genotypic or phenotypic variation, including genetic factors and external causes include various biological factors and abiotic factors such as temperature, food, and stocking density (Baras *et al*., 2011; Conner and White, 1999; Sukumaran *et al*., 2011; Umanah and Nlewadim, 2019). The formation of general individual size heterogeneity comes from the interaction of internal and external factors. In a more intuitive way, size heterogeneity facilitate cannibalism, but It's also affected by cannibalism, since the smallest fish are consumed by the largest ones (Kestemont *et al*., 2003). The existence of size heterogeneity contributed to the natural selection within the population (Conner and White, 1999). Cannibalism eliminated the individuals who were stunted by the above reasons of internal and external factors in the early stage of development, making the whole population develop away from recession and improving the survival ability of them (Baras *et al*., 2013; Ribeiro and Qin, 2016).

After animals are affected by some external factors, the internal environment will change accordingly, thus affecting the expression level of some genes (Gottlieb, 1998). Growth hormone/insulin-like growth factor (GH/IGF) system is the key promoter to regulate growth in vertebrates. In fish, the GH induces muscle growth by modulating the expression several of genes belonging to the GH, IGF systems, myostatin (MSTN) and atrophy (Fuentes *et al*., 2013). These genes have been shown to be closely related to growth in many fish (Gahr *et al*., 2008; Peterson *et al*., 2004; Picha *et al*., 2008), but do they contribute to size heterogeneity.

The immune system of fish has synergistic effects of

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0030-9923/2021/0001-0001 $ 9.00/0

innate immunity and acquired immunity, but the innate immunity is relatively developed. The innate immune system includes lysozyme, complement system and some cytokines (Kiron, 2012). The mechanistic target of rapamycin (mTOR) signaling pathway can regulate growth and homeostasis in an organism by perceiving and integrating multiple environmental cues (Laplante and Sabatini, 2012). Heat shock proteins (HSPs) are a group of stress proteins which can be triggered by environmental stress, such as starvation, hypoxia, water deprivation and microbial infection (Roberts *et al*., 2010). In raising barramundi juveniles, we found that comparing to their small siblings, larger fish from the same batch were likely to die sudden death when external factors changed. The resons are still unknown. In order to explore the causes of size heterogeneity and sudden death in juvenile fish, we explored the expression of growth and immune-related genes in three sizes of fish and inorder to correlate size heterogeneity and gene expression levels. Results from the present study will provide the background information on the size heterogeneity in artificial cultured fish.

MATERIALS AND METHODS

Incubation of fertilized eggs and larval rearing

Fertilized eggs from the same batch of barramundi broodstocks were obtained from the Tropical Fisheries Research and Development Center, Lingshui Town. Incubation of fertilized egg and for rearing of fish methods of Liu *et al*. (2018) were followed after entering the juvenile stage, all fish were transferred to 2000 L indoor tanks with the seawater recirculating aquaculture system. Stocking density was maintained at 0.2 fish L^{-1}. Fish were fed commercial diet twice a day at 0800 and 1600 h. Feces were cleaned up an h after each feeding. During the rearing period, the water parameters were measured daily and were maintained as ammonia nitrogen <0.1 mg L^{-1}, nitrite nitrogen <0.02 mg L^{-1}, pH 7.8, and dissolved oxygen >7.0 mg L^{-1}.

Grading and sampling

Ten weeks after rearing in hatchery, about 3000 juvenile fish were graded according to body length. In order to magnify the difference, the 0.15% fish were separated of the shortest and longest body lengths, respectively, and defined them as S grade and L grade, while the rest were M grade (Fig. 1). Nine fish were randomly sampled from each grade (body length and body weight in Table I). The fish were anaesthetized with 7 mg L^{-1} eugenol (Shangchi Dental Material Co., Ltd., Changshu, China) before handling and sampling. The tissues and organs sampled were brain, muscle, liver and kidney, which were snap-frozen in liquid nitrogen and then preserved in -80 °C until use.

Fig. 1. Different body length grades of barramundi (A, long grade; B, medium grade, C, short grade).

Table I. Body length and body weight sampled from all grades of barramundi.

Size grade	S	M	L
Body length (cm $fish^{-1}$)	2.19±0.19	4.05±0.22	9.15±1.27
Body weight (g $fish^{-1}$)	0.28±0.06	1.51±0.33	14.84±5.60

*S, short grade; M, medium grade; L, long grade.

The experimental procedure was complied with the standards of Institutional Animal Care and Use Committee guidelines (Suckow and Stewart, 2016). All experiments were conducted in line with the principles and guidelines for the care and use of live fish and the guidelines for animal experimentation approved by the Animal Experimental Council (AEC/NRIFS) of the National Research Institute of Fisheries Science, Fisheries Research Agency.

RNA isolation

The harvested frozen tissues (the brain, muscle, liver and kidney) were homogenized in 1 mL Trizol (Invitrogen and Greenprima Instrumenta Co., Ltd., UK), and RNA was separated in the chloroform layer and precipitated by isopropanol. The RNA pellet was washed in 1-mL 75% ethanol, and air-dried before resuspension in RNase-free water (Fu *et al*., 2019). The quantity of isolated RNA was later determined by measuring their absorbance at 260 and 280 nm using a ND 5000 spectrophotometer (BioTeke Corporation, China). Finally, the integrity of RNA was assessed using agarose gel (1%) electrophoresis.

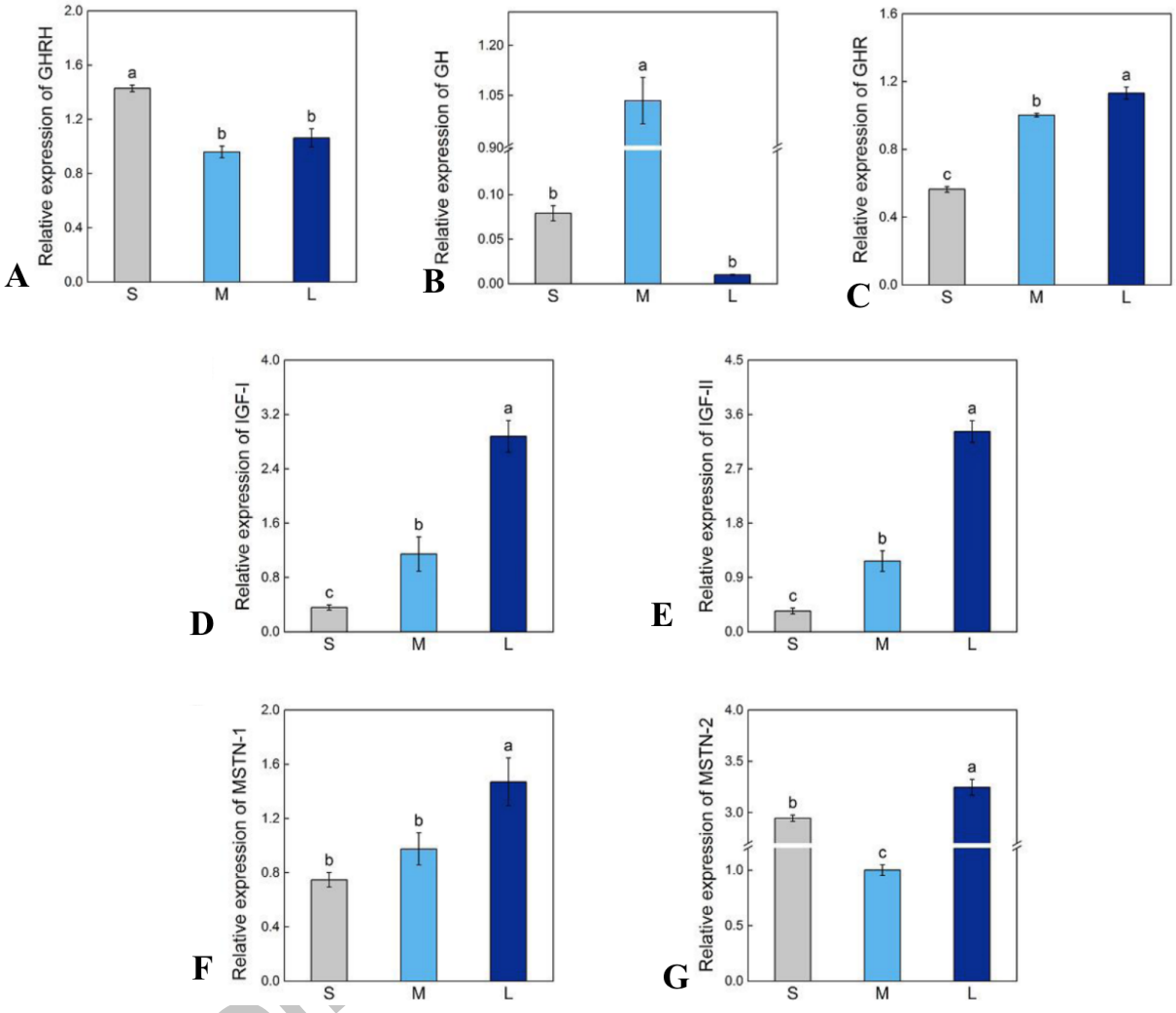

Fig. 2. Relative expression of growth-related genes(A-GHRH, B-GH, C-GRH, D- IGF-I, E- IGF-II, F- MSTN-1, G-MSTN-2)in different body length grades (S-Short grade, M- Medium grade, L-Long grade) of barramundi.
Different superscript letters indicate significant differences among grades ($P < 0.05$). Error bars represent standard error.

Genomic DNA removal and cDNA synthesis

The RNA was immediately used for cDNA synthesis. Subsequently, genomic DNA removal and reverse transcription was performed on 1 µg of total RNA using TransScript-Uni One-Step gDNA Removal and cDNA Synthesis SuperMix, that were determined according to the manufacturer's instructions using commercial kits (Transgen Biotech Co., Ltd., China). The synthesized cDNA samples were stored at -20 °C until further use.

Gene expression analysis

The genes chosen for analysis by qPCR were selected from the *L. calcarifer* NCBI database (https://www.ncbi.nlm.nih.gov/). The Primer Premier 5 program was used for designing the primers of growth hormone releasing hormone (GHRH), growth hormone (GH), growth hormone receptor (GHR), insulin like growth factor 1 (IGF-I), insulin like growth factor 2 (IGF-II), myostatin 1 (MSTN-1) and myostatin 2 (MSTN-2) mechanistic target of rapamycin (mTOR), eukaryotic translation initiation factor 4E (eIF4E), mTOR associated protein LST8 homolog (mLST8), heat shock cognate 70 kDa protein (HSP70), heat shock cognate 90 kDa protein (HSP90), tumor necrosis factor (TNF), interferon gamma 1 (IFN-γ1), interleukin-8 (IL8), interleukin-10 (IL10), transforming growth factor beta-1 (TGFβ1), C-reactive protein (CRP), complement C3 (C3) and complement C4 (C4) and β-actin (Table II). The qPCR was performed with the real-time

Table II. Primers of growth hormone releasing hormone (GHRH), growth hormone (GH), growth hormone receptor (GHR), insulin like growth factor 1 (IGF-I), insulin like growth factor 2 (IGF-II), myostatin 1 (MSTN-1), myostatin 2 (MSTN-2), mechanistic target of rapamycin (mTOR), eukaryotic translation initiation factor 4E (eIF4E) , mTOR associated protein LST8 homolog (mLST8), heat shock cognate 70 kDa protein (HSP70), heat shock cognate 90 kDa protein (HSP90), tumor necrosis factor (TNF), interferon gamma 1 (IFN-γ1), interleukin-8 (IL8), interleukin-10 (IL10), transforming growth factor beta-1 (TGFβ1), C-reactive protein (CRP), complement C3 (C3) and complement C4 (C4) and actin beta (β-actin) genes in barramundi used in qPCR.

Gene abbreviation	Primer sequence (5'-3')	Amplicon size(bp)	Accession no
GHRH	F: GCTGTTTTGTTGCCTGGTC	121	XM018681526
	R: CTGCTTCTCGGCTGGATTA		
GH	F: AGCCCCATTGACAAGCAC	86	X59378
	R: AACTCCCAGGACTCAACCAA		
GHR	F: AAGTCTGACCAATGGCAAGC	206	XM_018702498
	R: GCACCAAAGATGAGCAAAGC		
IGF-I	F: TGCCCTGCGGTACTAACCT	144	EU136176
	R: TGCCCTGCGGTACTAACCT		
IGF-II	F: AGTATTCCAAATACGAGGTGTG	131	XM_018664155
	R: GAAGATAACCTGCTCCTGTG		
MSTN-1	F: AACTGCGAATGAAAGAAGCTC	204	XM_018696695
	R: CTTGGACGATGGACTCAGGT		
MSTN-2	F: GTCTGTTCAGCCTCAGTCCA	145	XM_018661271
	R: CGGGTGTTGTTTCCCTCTTT		
IL8	F: TCTGACTGTTCCTGAGGCTATC	92	XM_018695863
	R: GACGTCCAATGGGCTTTCT		
IL10	F: TGCTGCCGTTTTGTGGAG	194	XM_018686737
	R: ACCGTGCTCAGGTAAAAGTCC		
TGFβ1	F: TACCTCGCTTCCCGTTTC	105	XM_018665504
	R: CTGCTCATCCTCAGTCCCTC		
TNF	F: AAGGACTCCGCTGAGAAAAC	241	XM_018699809
	R: TGAACGATGCCTGGCTGTA		
IFN-γ1	F: TACCAGGAGCAGGACAAGC	134	NM_001360734
	R: TCGTCAGGCAGCGAACTT		
CRP	F: ACCGAACTGAAGACCACGAT	106	HQ652974
	R: TGGGGCACCTCAAACAAA		
C3	F: AAATGCTGCCATCGTTCC	175	XM_018679796
	R: CCAGTGACCTTCAGACCAAA		
C4	F: CGAGGTTGAACGAAAAGGAC	97	XM_018688206
	R: CACAGCAAGCAAAGCCACT		
mTOR	F: GTTTCTTCCGCTCCATTTC	110	XM_018675222
	R: CAGGGCTTCATTCACTTCA		
mlst8	F: TGATTCAACACTATTAGCCACA	212	XM_018687802
	R: TTTCCACGCACCACAGG		
eIF4E	F: TGACGACTACAGCGATGAT	183	XM_018697729
	R: GTGTCTGCGTGGGATTG		
HSP70	F: CTGGAGTCCTACGCTTTCAA	204	HQ646109
	R: CTTGCTGATGATGGGGTTAC		
HSP90	F: ACGATGATGAGCAGTATGCC	201	XM018661637
	R: CAAACAGGGTGATGGGGTA		
β-actin	F: AACCAAACGCCCAACAACT	112	XM_018667666
	R: ATAACTGAAGCCATGCCAATG		

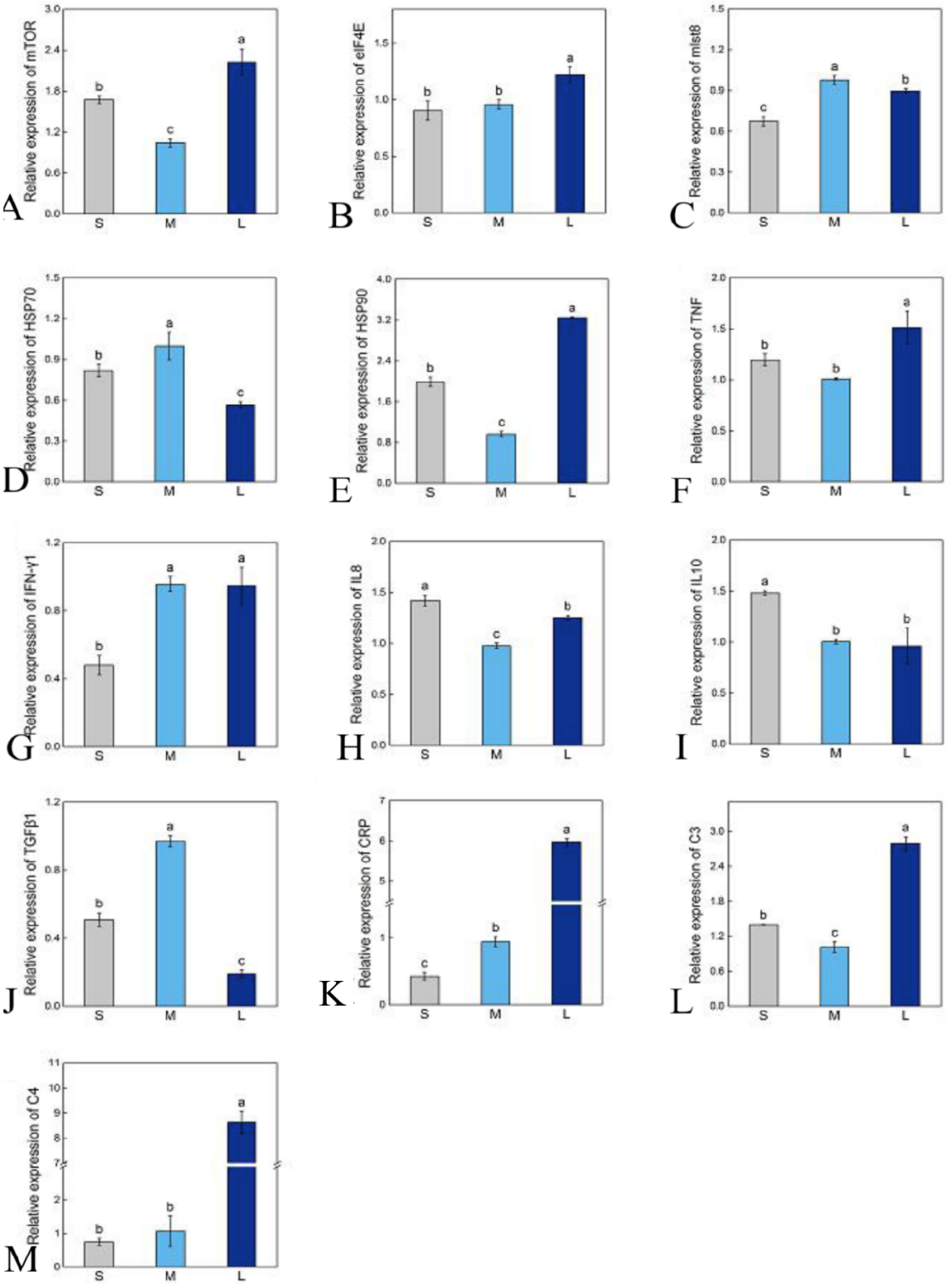

Fig. 3. Relative expression of immune-related genes(A-mTOR, B-eIF4E, C-mLST8, D- HSP70, E-HSP90, F-TNF, G-IFN-γ1, H-IL8, I-IL10, J-TGFβ1, K-CRP, L-C3, M-C4) in different body length grades(S-Short grade, M- Medium grade, L-Long grade) of barramundi.

Different superscript letters indicate significant differences among grades ($P < 0.05$). Error bars represent standard error.

qPCR analysis (Hangzhou Longgene Scientific Instrument Co., Ltd., China) using SYBR Green (Tiangen Biotech Co., Ltd., China). The 20 µl of reaction including 10 µl 2×RealUniversal PreMix, 0.6 µl of each primer (10 µM) and 2 µl of diluted cDNA was initially denatured at 95 °C for 15 min and then amplified for 40 cycles (95 °C, 10 s, 58 °C, 20 s and 72 °C, 30 s). All steps are carried out according to the manufacturer's instructions. For each sample, the PCR reactions were performed in triplicate.

At the end of each RT-qPCR cycle, the melting curve analysis of the primers was performed to ensure only specific products were obtained with no formation of primer dimers. No template control was included with each assay to verify that PCR master mixes were free of contamination. After verification of PCR efficiency to be around 100%.

Calculations and statistical analysis

The $^{\Delta\Delta}$Ct method was used to calculate the relative expression with β-actin as a reference gene, and normalized to control (Green and Sambrook, 2012). The data were expressed as the mean ± standard deviation (SD). Statistical analyses were carried out by PASW Statistics (version 18). Comparisons between different groups were conducted by one-way ANOVA and LSD test, and significant difference was set at $P < 0.05$.

RESULTS

The expression of growth-related genes

The GHRH expression in the S grade and the GH gene expression level in the M grade was significantly upregulated (Fig. 2A, 2B, $P < 0.05$). There were significantly different in expression levels in three grades of GHR, IGF-I, IGF-II and MSTN-1 ($P < 0.05$), while the expression levels were upregulated with the increasing of body length (Fig. 2C, D, E, F). The MSTN-2 expression was significantly affected by the size grade ($P < 0.05$), and the expression upregulated in L grade and downregulated in M grade (Fig. 2G).

The expression of immune-related genes

In the mechanistic target of rapamycin (mTOR) signaling pathway genes of liver, the mTOR expression in the S grade and the L grade were significantly up-regulated compared to the M grade ($P < 0.05$), and the expression level in L grade was significantly higher than those in S grade (Fig. 3A). The elf4e expression in the L grade was significantly up-regulated compared to the M grade ($P < 0.05$), while the S grade and the M grade showed no significant difference (Fig. 3B, $P > 0.05$). The mLST8 expression in the S grade and the L grade were significantly down-regulated compared to the M grade ($P < 0.05$, Fig. 3C).

In heat shock protein genes, the HSP70 expression in the S and L grade HSP70 were significantly down-regulated compared to the M grade ($P < 0.05$), and the lowest expression level was observed in L grade (Fig. 3D). The HSP90 expression in the S and L grade was significantly up-regulated compared to the M grade ($P < 0.05$), and the highest expression level was observed in the Le (Fig. 3E).

In cytokine genes, the expression of TNF was significantly up-regulated in the L grade compared with the other two grades ($P < 0.05$), and there was no significant difference between the S and M grade (Fig. 3F, $P > 0.05$). In the expression of IFN-γ, the S grade was significantly down-regulated compared to the other two grades ($P < 0.05$), while there was no significant difference between the M and L grade (Fig. 3G, $P > 0.05$). The S grade in IL8 and IL10 was significantly up-regulated compared to the other two groups, and the highest expression of IL8 was observed in L grade (Fig. 3H, I, $P < 0.05$). In the expression of TGF, both the S and L grade were significantly down-regulated compared to the M grade ($P < 0.05$), and the lowest expression was observed in L grade (Fig. 3J).

In inflammatory marker genes of liver, CRP expression level was significantly up-regulated by body length (Fig. 3K, $P < 0.05$). In complement system genes, the C3 and C4 expression in the L grade were significantly up-regulated compared to the other two groups, and the S grade was significantly up-regulated compared to the M grade in C3 (Fig. 3L, M, $P < 0.05$).

DISCUSSION

In teleost fishes, as in other vertebrates, growth is under the control of the growth hormone/insulin-like growth factor (GH/IGF) system, as well as insulin and other endocrine and local factors (Pierce *et al.*, 2005; Rolland *et al.*, 2015). In this study, the downstream genes of the growth axis, such as GHR, IGF-I and IGF-II, showed a significant increasing trend with the increase of body length. The expression of IGF-I and IGF-II genes is positively correlated with growth performance, that was observed in previous studies of clownfish (*Amphiprion ocellaris*) (Avella *et al.*, 2009) and golden pompano (*Trachinotus ovatus*) (Tan *et al.*, 2017). GH plays its role of biological actions attributed to the production of IGF-I when bound with GHR (de Azevedo Figueiredo *et al.*, 2007). Although GH is a universal growth regulator, its regulation of growth rate is delayed and has negative feedback regulation with IGF-I (Gabillard *et al.*, 2006). These reasons may be explained why GH did not show regular changes with body

length grade in this study. GHRH is specially interacted with the GHRH receptor (GHRHR) to have contributed to stimulating GH synthesis and releasing from anterior pituitary cells (Nam *et al*., 2011). In this study, there was no consistency between the expressions of GHRH and GH. The neuroendocrine regulation of GH secretion in fish has been recognized as multifactorial, with a balance between stimulatory and inhibitory factors acting on the somatotrope (Canosa *et al*., 2007). Further work may need to be conducted to fully assess the control of GH expression in different body length grades of barramundi.

Myostatin is an identified member of the transforming growth factor-β (TGF-β) superfamily (Østbye *et al*., 2001). In teleost fishes, MSTN-1 appears to primarily inhibit muscle hyperplasia, but perhaps not hypertrophy, and the down-regulation of MSTN-2 is the main cause of grown through muscle hypertrophy (de Santis *et al*., 2012). However, there was no inhibition of growth at the expression level in this study, suggesting that the barramundi may not have entered a period of rapid muscle hyperplasia at this stage (Johnston, 1999).

The mechanistic target of rapamycin (mTOR) signaling pathway can sense and integrate a variety of environmental cues to affect energy metabolism protein synthesis and lipid synthesis, thus regulating homeostasis and growth in an organism (Laplante and Sabatini, 2012). The mTOR is known as an atypical serine/threonine protein kinase and has the ability of forming two distinct complexes named mTOR complex 1 (mTORC1) and 2 (mTORC2) by interacting with several proteins. The mTORC1 directly phosphorylates eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) and S6 kinase 1, the translational regulators, which, in turn, promote protein synthesis (Ma and Blenis, 2009). The mLST8 is involved in both mTORC1 and mTORC2, and it is necessary for the mTORC2, but its activity shortage does not affect the activity of mTORC1 (Jacinto *et al*., 2004; Kim *et al*., 2003). In this study, mTOR pathway related genes were relatively active in L grade, and there was no abnormal up-regulated expression, indicating that L grade was relatively active in protein translation, metabolism and other aspects.

In aquatic animals, HSPs associated with the host response to environmental pollutants and food toxins, particular in the specific and non-specific immune responses to bacterial and viral infections and the development of inflammation, so they have been shown to play a significant role in health (Basu *et al*., 2002). HSPs was less expressed in the absence of environmental stimulation (Roberts *et al*., 2010). In this study, there was no consistent rule between the body length levels of HSP70 and HSP90, which may be the expression level fluctuated in a low range or that was not correlated with body length.

Pro-inflammatory cytokines and anti-inflammatory cytokine balance is maintained between the body's normal immune status, disease resistance, its stability and normal physiological activities of the key factors, inflammatory cytokines to activate the body is mainly responsible for the pathogens, congenital and acquired immune system, to destroy the intruder, and anti-inflammatory cytokine mainly was eliminated after the intruder to eliminate inflammation, make the body return to normal immune and physiological level (Bird *et al*., 2006; Secombes *et al*., 2001). In this study, pro-inflammatory cytokines (TNF and IL8) were up-regulated, but anti-inflammatory cytokine (TGFβ1) was significantly down-regulated in L grade. This is not conducive to balance between cytokines, the risk of inflammatory disease would be increased.

The CRP is an important inflammatory marker and it has a pleiotropic effect. Both "pro-inflammatory" and "anti-inflammatory" activities have been described (Black *et al*., 2004). The CRP is capable of activating complement, opsonizing bacteria, fungi and parasites and agglutinating particles (Ballou and Lozanski, 1992). Complement proteins C3 and C4 are also classified as acute phase reactants as their synthesis is upregulated during inflammation (Watts *et al*., 2001). In this study, the expression levels of CRP, complement gene C3 and C4 in the L grade were uniformly up-regulated, it further confirm the existence of inflammatory response in grade L, which may be the reason for its reduced tolerance to the environment

CONCLUSIONS

Collectively, our gene expression analyses suggest that the expression level of GHR, IGF-I and IGF-II is consistent with body length, but there is no similar rule between GHRH, GH, MSTN-1 and MSTN-2. TOR pathway related genes were more actively expressed in L grade. Heat shock protein has no obvious rule in body length grade. L grade pro-inflammatory cytokines were up-regulated, but anti-inflammatory cytokines were down-regulated and inflammatory markers were up-regulated, indicating an obvious inflammatory response. The expression levels of S and M grade anti-inflammatory and proinflammatory cytokines was more balanced. Our results would pave the way to further understand of the relationship between size heterogeneity and gene expression in barramundi juveniles.

ACKNOWLEDGEMENTS

This work was supported by Science and Technology Innovation Team Project of Chinese Academy of Fishery

Sciences [grant no. 2020TD55], Central Public-interest Scientific Institution Basal Research Fund, South China Sea Fisheries Research Institute [grant no. CAFS-2017ZD01, CAFS-2018ZD01], Guangxi Innovation Driven Development Special Fund Project [grant no. Guike AA18242031], and Key Research and Development Project of Hainan Province [grant no. ZDYF2017036, ZDYF2018096].

Statement of conflict of interest

The authors have declared no conflict of interest.

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