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Physiological Response of Asian Seabass Reared in Recirculating Aquaculture System under Different Stocking Densities

Selvaram Ezhilmathi[1], Baboonsundaram Ahilan[1], Arumugam Uma[1], Nathan Felix[2], Antony Cheryl[1], Ramasamy Somu Sunder Lingam[3], Nallaiah Hemamalini[4]

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ABSTRACT

Background: There is a gap that we will need to use innovative solutions to produce more food and nutrition. RAS is a continuous water-flowing system that can induce schooling behaviour, a phenotypic character seen in Asian seabass that could help cut down cannibalism at a certain level. Stocking density is one of the critical factors affecting the growth, survival and health status of animals, which can influence the antagonistic behaviour pattern, hierarchical phenomena and cannibalistic nature of Asian seabass during the early life stage.

Methods: A 60-day trial was conducted to evaluate the effect of different stocking densities (70, 140, 210, 280 and 350 fish/m^3) on biochemical and stress gene expression of Asian seabass (*Lates calcarifer*) reared in Recirculating Aquaculture System by following a completely randomized design with four replications.

Result: The study found that fish reared at 350 fish/m^3 had significantly higher haemoglobin and red blood cells. In the case of biochemical parameters, total protein, Albumin and Globulin were lower in 70 fish/m^3 and Blood Urea Nitrogen, cholesterol and triglycerides were significantly higher in 350 fish/m^3 treatment. The relative gene expression of HSP70, HSP90A and GST was significantly upregulated with the increasing stocking density. The study suggests that the rearing of Asian seabass at a stocking density of 70 fish/m^3 in a recirculatory aquaculture system could improve the growth performance and metabolic and molecular activities of the fish.

Key words: Asian seabass, Biochemical parameter, HSP70, HSP90A, GST.

INTRODUCTION

Fisheries and aquaculture demonstrated their significant role in providing food, nutrient and employment. Aquaculture has already demonstrated its critical role in global food security and production (7.5% growth rate since 1970). The enormity of the environmental challenges the sector must face as it intensifies production, demands and new sustainable aquaculture development strategies. There is a gap that we will need to use innovative solutions to produce more food and nutrition. Culture technology, feed and species selection is the most important factor controlling sustainable aquaculture production. Application of advanced culture systems like Recirculating aquaculture system (RAS), IMTA and biofloc technology is very useful with a combination of maximum production capacities. Barramundi is currently being explored as a potential candidate for such systems, using marine, brackish, or freshwater (Harpaz *et al.* 2005). The Asian seabass fetches a high market price due to its delicately flavoured white meat and is an economically suitable species for the RAS technology.

Stocking density, a critical production determining factor, affects the growth, survival and welfare of fish under captive conditions (Ashley, 2007; Ezhilmathi *et al.* 2022) and that can influence the antagonistic behavior pattern, hierarchical phenomena and cannibalistic nature of Asian seabass during the early life stage (Mojjada *et al.* 2013). Asian seabass juveniles compete for food and space in controlled conditions, leading to size variation and promoting cannibalism (Khan *et al.* 2021). RAS is a continuous water-flowing system that can induce schooling behavior, a

[1]Department of Aquaculture, Dr. M.G.R. Fisheries College and Research Institute, Ponneri-601 204, Tamil Nadu, India.
[2]Directorate of Incubation and Vocational Training in Aquaculture, Tamil Nadu Dr. J. Jayalalithaa Fisheries University, ECR-Muttukadu, kancheepuram-603 112, Tamil Nadu, India.
[3]Krishnagiri-Barur Centre for Sustainable Aquaculture, Tamil Nadu Dr. J. Jayalalithaa Fisheries University, Barur, Nagapattinam-611 002, Tamil Nadu, India.
[4]Institute of Fisheries Post Graduate Studies, Vaniyanchavadi-603 103, Chennai, India.

Corresponding Author: Selvaram Ezhilmathi, Department of Aquaculture, Dr. M.G.R. Fisheries College and Research Institute, Ponneri-601 204, Tamil Nadu, India.
Email: s.ezhilmathi.mfsc@gmail.com

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phenotypic character seen in Asian seabass (Anwar *et al*. 2016) that could help cut down cannibalism at a certain level. In this context, it is important to study the growth and stress of the Asian seabass reared in RAS under different stocking densities for further sustainable expansion of this species culture in the RAS-based system.

Materials and Methods

Experimental design

The study was conducted in a RAS at the Advanced research farm facility, Madhavaram, Tamil Nadu (13°09′34.4″N 80°14′55.3″E). The experimental tank was supplied with fresh water and operated with a recirculation rate of 90%, a moderate flow-through rate that allowed water renewal to the RAS eleven times per day at approximately 3 L/min. Twenty numbers of 500L circular FRP tanks were used indoors. The water was continuously aerated using air ventures connected to the tank inlet. 5,000 numbers of hatchery-bred juvenile Asian seabass were procured from the Central Institute of Brackishwater Aquaculture (CIBA) and acclimatized in 2,000L FRP nursery tanks. After sizing, fish (5.20±0.10 g) were stocked randomly in a 500L capacity tank, connected with RAS, at five different treatment stocking densities *viz.,* T1 (70/m^3, 350 g/m^3), T2 (140/m^3, 700g/m^3), T3 (210/m^3, 1050 g/m^3), T4 (280/m^3, 1400 g/m^3) and T5 (350/m^3, 1750 g/m^3) and each treatment had four replications. Fish were daily fed to *ab-libitum* with commercial floating feed (50% protein, 10% lipid, 1% crude fiber, 17.7% ash and 7% moisture). Uneaten feed was collected, after 1h of feeding, by scoop net and it was dried and weighed.

Quantitative real-time PCR (qRT-PCR)

At the end of the culture, total RNA was extracted from the collected muscle tissues using an RNA iso-plus kit (Takara Bio Inc. Japan) according to the manufacturer's protocol. First strand cDNA was synthesized from 2 μg of total RNA using the first-standard cDNA synthesis kit (Thermo Scientific, USA), the first-standard complementary DNA (cDNA). The relative gene expression study was carried out following the standard method (Michelato *et al*. 2017) (Table 1). β-actin transcript, as an internal control, was used to study the quantitative real-time-polymerase chain reaction (qRT-PCR). The qRT-PCR was carried out in a C1000 Touch thermal cycler-CFX96 Real-time PCR (Bio-Rad, USA). For gene expression study, specific primers were designed and used. qRT-PCR was performed using 20 ng of cDNA template, 10 μM of each primer (forward and reverse) and 1x SYBR Green PCR Master Mix Kit (Takara Bio Inc. Japan) and nuclease-free water to make a total volume of 20 μl. The qRT-PCR cycle threshold (Ct) values were measured and a relative expression level of the specific gene was presented as $2^{-\Delta\Delta Ct}$.

Heamto-Biochemical analysis

The haematological parameters such as the content of red blood cells (RBC), haemoglobin (Hb), haematocrit (Ht), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were analyzed using 3-Part Hematology Analyser (M/s. Meril Diagnostic Private Ltd.). The serum biochemical parameters, namely total protein (TP), glucose (GLU), cholesterol (TC) and triglycerides (TG) levels, were analyzed by an automated biochemistry analyzer - A-15 Biochemical Analyser (M/s. Biosystems, Sriperumbudur).

Statistical analysis

All data were presented as mean values ± standard error of the four replicates. The data were statistically analyzed in SPSS 22.0 using One-way ANOVA to find the significant difference among the treatment mean values. Duncan's test for multiple comparisons was used to rank the mean values if the data significantly differed ($p<0.05$).

Results and Discussion

Growth related gene expression

Fish growth was affected by internal and external factors such as temperature, nutritional requirement, photoperiod, *etc*. (Moriyama *et al*. 2000). The physiological responses were processed and controlled by the brain-hypothalamus and pituitary gland in fish. The GH/IGF-1 axis is important in regulating fish physiology (Long *et al*. 2019), such as cell proliferation and differentiation, protein synthesis and tissue maintenance (Patel *et al*. 2005). The MSTN is a member of the Transforming Growth Factor Beta (TGF-β) cytokine superfamily, which negatively regulates muscle growth by inhibiting the muscle satellite cell differentiation during early development (Artaza *et al*. 2005) and adult (Welle *et al*. 2007). Picha *et al*. (2014) reported a positive correlation between somatic growth and IGF-1, which acts as a biomarker of fish growth. In this study, the relative gene expression of IGF-1, GH, MSTN, HSP70, HSP90A and GST were expressed in fold changes by considering lower stocking density (T1 group) as one-fold. Relative growth

Table 1: Primers used for qRT-PCR to relative gene expression analysis of asian seabass.

Gene name	Primer sequence (5'-3')
IGF-1	F-CAGTGGCATTTATGTGATGTCTTC
	R- TGAGGACGCACAGCAGTAG
Growth hormone	F-TCGACAAACACGAGACGCA
	R-CCCAGGACTCAACCAGTCCA
Myostatin-1	F-ATGTAGTTATGGAGGAGGATG
	R- CTTGGACGATGGACTCAG
β-actin	F-TACCACCGGTATCGTCATGGA
	R- CCACGCTCTGTCAGGATCTTC
HSP70	F- CAAGGTGATTTCAGATGGAGG
	R- CTTCATCTTCACCAGGACCA
HSP90A	F-AGAAAGAAGTGGACCTTGAG
	R-CTTTGTGTCTTCATCCTCGT
GST	F-GTAATTCAAGATCGCCTTTGTC
	R-TTAACAGTTGCAGAAGTGGAG

gene expression is shown in Fig 1 and stress gene expression in Fig 2. The study found a clear trend with a significant difference in gene expression of different stocking densities reared fish. In muscle, relative growth gene expression of GH/IGF-1 and MSTN were downregulated and upregulated, respectively, with an increase in stocking density. Relative stress gene expression of HSP70, HSP90A and GST were upregulated in muscle with an increase in stocking density. Relative GH/IGF-1 (1.094 ±0.012 folds and 1.075±0.023folds) and MSTN (2.183±0.011 folds) expressions were significantly higher in T1 and T5, respectively, groups reared fish. The GH and IGF-1 expressions were significantly lower in T5 (0.105±0.003 folds and 0.506±0.003 folds), respectively, in reared fish.

On the other hand, MSTN expression was significantly lower (1.004±0.001 folds) in the T1 group. The lower growth performance in the T5 group was accompanied by the changes in the expression of GH/IGF-1 and MSTN in muscle and stress created by the higher stocking density. In fine flounder (*Paralichthys adspersus*), stocking density directly affected the growth and down-regulated the GH/IGF system (Mendez *et al.* 2018). Another possible explanation for decreased growth performance in the high stocking density groups of Asian seabass might be the less feed intake under crowding stress, which upregulated the fish muscle transcripts of MSTN. There is a negative relationship between growth and stocking density, which could be due to energy imbalance and less nutrient digestion and

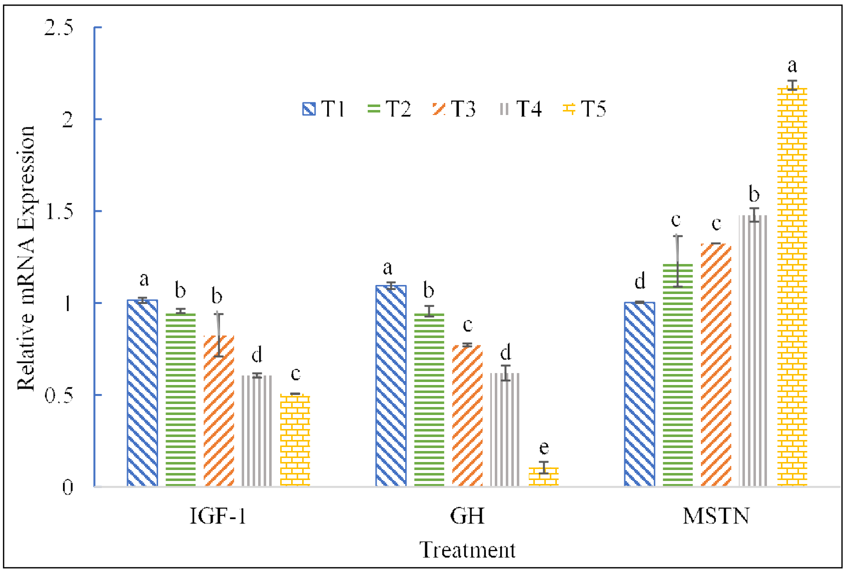

Fig 1: Relative mRNA transcript levels of GH/IGF-1 and MSTN in the muscle of Asian seabass maintained in different stocking densities over a 60-day period. Values represent the mean±SEM. Different letters indicate statistically significant differences (P<0.05).

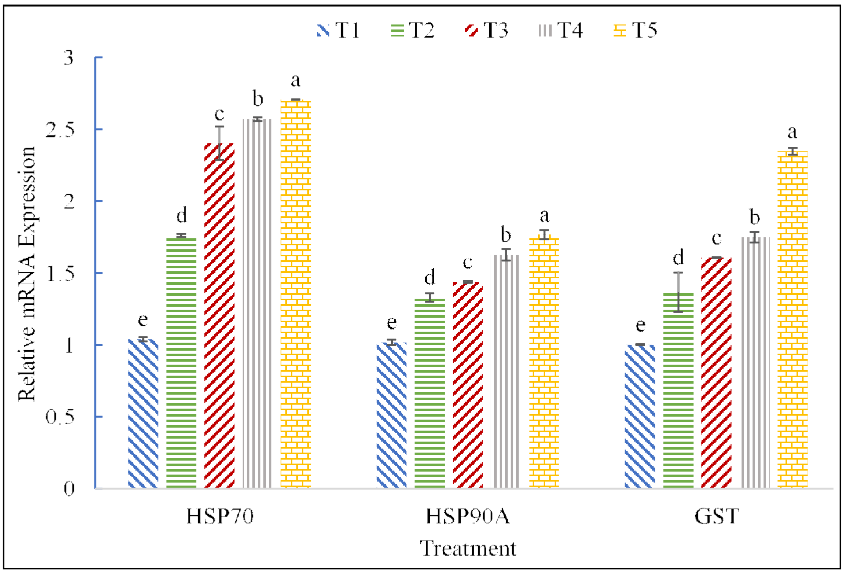

Fig 2: Relative mRNA transcript levels of HSP70, HSP90A and GST in the muscle of Asian seabass maintained in different stocking densities over a 60-day period. Values represent the mean±SEM. Different letters indicate statistically significant differences (P<0.05).

absorption of fish during crowding stress. In Amur sturgeon (*Acipenser schrenckii Brandt*), the GH/IGF-1 expression in the muscle was down-regulated with increasing stocking density (5.5 kg/m^3 to 11.0 kg/m^3), but stocking density had not influenced the IGF-2 expression (Ren *et al*. 2018); IGF-1 and MSTN were significantly downregulated and upregulated with high stocking density (12 to 44 kg.m^3), respectively, in rainbow trout (*Oncorhynchus mykiss*). Similarly, in *Eleginops maclovinus,* GH/IGF-1 expression was significantly reduced in high stocking density (24 kg/m^3) due to increased biomass and crowding stress (Oyarzún *et al*. 2020).

Heat shock protein is also known as stress proteins, a highly conserved family of cellular proteins that act as molecular partners in all organisms and play an important role in fish stress (Qiang *et al*. 2015; Zahedi *et al*. 2019). Altered expression of HSP 70 andHSP90 in muscle tissue is a common biomarker as the elevated level is correlated with an elevated level of energy requirement and demotes the growth of Asian seabass same kind of results were observed in the Atlantic salmon and rainbow trout (Bower and Johnston, 2010; Galt *et al*. 2018). GST belongs to the primarily soluble enzymes that have important functions in detoxification and antioxidation caused by the action of ROS (O'Brien *et al*. 2000). Relative expression of HSP70, HSP90A and GST was significantly higher in T5 groups reared fish. The HSP70 (1.038±0.140 folds), HSP90A (1.017±0.094 folds) and GST (1.001±0.025 folds) expressions were significantly lower in T1 reared fish. Increased HSP90 expression in muscle tissue in response to stress is hypothesized to protect muscle proteins from degradation and possibly promote protein synthesis or recycling (Naito *et al*. 2000; Goto *et al*. 2003). Herein, crowding increased HSP90 in white muscle in cutthroat trout, brook trout and Atlantic salmon induced HSP90 elevation observed in other teleost tissues (Vijayan *et al*. 2003). The GST mRNA level in the muscle was significantly downregulated with increasing the stocking density and similar kinds of results were recorded in turbot (Chan 1995) and Chinese sturgeon (*Acipenser Sinensis*) (Long *et al*. 2019). The study found significantly elevated relative gene expression of GH/IGF-1 in the T1 group and this could be due to optimal nutrient digestion and absorption with a sufficient level of protein-sparing effect in fish to balance the energy required to overcome crowding stress.

Heamatologicl parameters

Haematological parameters are general indicators of fish health (NRC, 1993). Haematological characteristic is an important tool that can effectively monitor physiological and pathological changes in fishes. Normal ranges for various blood parameters in fish have been established by different researchers in various conditions, including normal fish physiology, stress and pathological condition (Kumar *et al*. 2017). van Rijn (1996) revealed that the glucose level in serum might improve with the elevation of stress. The haemato-biochemical response in Asian Seabass towards different stocking densities is shown in Table 2. The present study results revealed that increasing stocking density creates chronic stress in the animal and decreases growth. Blood glucose levels might be affected by the high stocking density, capture and acute stress factors (Luo *et al*. 2013). It acts as a transient indicator of the stress level; when stress is continuous, the blood glucose level falls to a pre-existing level. A previous study of *Pagrus pagrus* (61.1±1.35mg/dl) (Rotllant *et al*. 1997) and rainbow trout (5.1±0.37 mmol/L) (Galt *et al*. 2018) suggested that chronic high-density stress has a limited effect on serum glucose levels in fish. In the present study, at a lower stocking density (70 fishes /m^3), glucose level was found to be 58.6±0.22 mg/dl, which increased with increasing stocking density (134.63±0.25 mg/dl). Fotedar (2016) also reported similar results in Asian seabass, where the glucose level at low stocking density was (4.3±0.45 mmol) and at higher stocking density 9.8±0.03 mmol/L, which almost changed the metabolic activity of the fish and decreased the growth rate. Serum total protein level is also used as an indicator of fish health (Tahmasebi-Kohyani *et al*. 2012). Higher-level Hb (10.4±0.05 mg/dl) and RBC (4.17 $\times$ 106±0.02 microliter) values were observed at higher stocking density (210 fish/m^3) could be attributed to the increasing the haemoconcentration, which was

Table 2: Heamato-biochemical parameters of asian seabass reared in different stocking density.

Parameter	T1	T2	T3	T4	T5
HB (g/dl)	4.35±0.05^e	4.93±0.05^d	5.13±0.03^c	8.7±0.05^b	10.4±0.05^a
RBC (million/cum)	1.42±0.02^e	1.48±0.01^d	1.82±0.02^c	3.62±0.01^b	4.17±0.02^a
PCV (%)	12.78±0.18^e	14.7±0.38^d	17.53±0.29^c	35.15±0.2^b	38.1±0.23^a
MCV (ft)	93.93±0.25^b	96.88±0.43^a	91.28±0.18^c	93.8±0.25^b	88.73±0.39^d
MCH (pg)	28.38±0.21^c	25.13±0.4^d	25.85±0.37^d	29.83±0.34^b	37.45±0.45^a
MCHC (g/dl)	30.78±0.45^c	25.98±0.41^e	28.23±0.26^d	32±0.34^b	42.35±0.41^a
Blood glucose (mg/dl)	58.56±0.22^d	78.14±0.32^c	134.63±0.25^a	95.85±0.51^b	77.93±0.3^c
Blood urea Nitrogen (mg/dl)	15.06±0.31^a	10.36±0.28^c	12.51±0.26^b	11.64±0.39^b	8.16±0.38^d
Cholesterol (mg/dl)	156.1±0.47^b	106.58±0.17^e	121.85±0.4^d	131.4±0.47^c	157.48±0.28^a
Triglycerides (mg/dl)	156.68±0.21^e	169.13±0.38^c	167±0.4^d	172.85±0.39^b	182.83±0.32^a
Total protein (g/dl)	6.27±0.27^a	4.82±0.43^b	6.38±0.31^a	4.43±0.35^{bc}	3.43±0.33^c

Data are mean values of four replicates ±SEM (n = 4). Means in the same row with different superscripts are significantly different ($p<0.05$).

similar to the study of Rotllant *et al*. (1997) in *Pagrus pagrus*. The MCH and MCHC play a significant role in diagnosing anaemia in most animals and are known to indicate the erythrocyte status and oxygen-carrying capacity of the blood in fish (Houston, 1997). An increase in the number of cells could relate to the increase in the respiratory demand in the higher stocking density (Shen *et al*. 1991; Zhou *et al*. 2008).

CONCLUSION

The relative expression of the GH/IGF axis and MSTN indicated that crowding stress influenced growth by regulating growth gene expression. The relative expression of the HSP70, HSP90A and GST indicated crowding stress in fish. Overall, the present experimental results suggest that Asian seabass could be reared in the RAS system with a maximum stocking density of 70 fish per m^3 without any negative impacts on its growth and physio-metabolic activities.

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Supplementary data

Supplementary data for this article can be found in online by following link https://doi.org/10.1111/are.15725.

Conflict of interest: None.

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