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How different temperatures and feeding rates impact physiological and histological responses of juvenile Asian seabass (*Lates calcarifer*)

Rosa Aghaei Meybodi[1], Mahmood Nafisi[1, 2], Ahmad Ghasemi[2], Reza Davoodi[1], Vahid Morshedi[2], Ebrahim Sotoudeh[1], and Ghasem Rashidian[3]

ABSTRACT

The current study evaluated the interactive impacts of water temperature and feeding rate on digestive enzymes, intestine histology, growth and stress-related genes, and cultivable intestinal microbiota of Asian seabass (*Lates calcarifer*). For this purpose, 180 fish (85.0±3.0 g) were reared at three different temperatures (20, 27, and 33°C) and two feeding rates (apparent satiation and 2.5% of biomass) with three replications for 6 weeks. The results revealed no significant differences among different treatments regarding the activity of digestive enzymes (P> 0.05) of fish reared under different temperatures and feeding rates. The length, width, and thickness of intestinal villi were unaffected by different temperatures and feeding rates (P>0.05). In addition, no variations were found in the total aerobic bacterial count of fish gut from different experimental groups (P> 0.05). At the molecular level, *IGF-I* and *HSP70* coding genes were found to be highly expressed in experimental treatments (P< 0.05). To conclude, the present study showed that temperatures between 27 to 33°C are more optimal for Asian seabass, and the different temperatures and feeding rates do not affect digestive enzymes, intestine histology, and gut microbiota after 6 weeks.

Keywords: Temperature, Feeding rate, Digestive enzymes, Gut microbiota, Gene expression, Asian seabass.

1 Department of Fisheries, Faculty of Nano and Bio Science and Technology, Persian Gulf University, Bushehr, Islamic Republic of Iran.
2 Persian Gulf Research Institute, Persian Gulf University, Bushehr, Islamic Republic of Iran.
3 Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Institute of Aquaculture and Protection of Waters, University of South Bohemia in České Budějovice, Husova třída 458/102, 370 05 České Budějovice, Czech Republic.
*Corresponding author; e-mail: v.morshedi@gmail.com

INTRODUCTION

Fish live in a 3-dimensional complex aquatic environment, and since fish are poikilothermic animals, surrounding temperature is a major factor that regulates fish's metabolism and growth rate (Fry, 1971; Groot et al., 1996). Fish are believed to perform best at their optimum temperature, which varies even for different life stages of one species (Pedersen and Jobling, 1989), where feed utilization efficiency is maximum. Thus, for the best nutrition management, aquafeed manufacturing companies often provide a range of feeding rates according to water temperature for each specific growing stage of the target species. Therefore, knowing the optimum temperature for each species can help with efficient production and increase the overall yield. It is important to mention that feed accounts for 50 to 80% of the total aquaculture production costs. Moreover, underfeeding or malnourishment might render fish more susceptible to diseases and result in mass mortalities, while overfeeding results in feed loss and water quality deterioration, making aquaculture practice less profitable.

The size of the fish and water temperature are important factors for optimizing feeding rates (Kestemont and Baras, 2001; Wang, Xu, and Kestemont, 2009). For instance, the best growth performance of pike perch (*Sander lucioperca*) was obtained when fish were fed to apparent satiation (Ming *et al.*, 2013). Given that, the rearing temperature and feeding rate are closely interlinked and affect various physiological processes of fish. The impacts of feeding rate and different rearing temperatures on various physiological aspects of fish have been reported by other researchers (Fang *et al.*, 2010; Kim *et al.*, 2014; Baloi *et al.*, 2017; Volkoff and Rønnestad, 2020). Both the feeding rate and the feeding time greatly influenced the activity of digestive enzymes. Moreover, several factors, such as enough enzyme levels and time for digestion and absorption, can affect the digestion process (Harpaz *et al.*, 2005). Furthermore, water temperature can affect digestive enzyme activities by affecting evacuation time (Temming and Herrmann, 2001).

Asian seabass (*Lates calcarifer*), an important food fish in tropical regions bordering the Indian Ocean, has been extensively studied due to its economic significance (Jerry, 2013). Asian seabass is cultivated in different aquaculture systems and represents a prospective option for diversifying

coastal aquaculture of marine fish. According to previous studies, the fish can reach their growth potential when fed below or at an apparent satiation level, depending on the species (Han *et al.*, 2004*;* Fang *et al.,* 2010). In addition, the increase in temperature can impact the digestive system, intestinal microbiota, and, consequently, growth performance. However, the combined effects of rearing temperature and feeding rate on have not been well studied in Asian seabass. Thus, in this study, we evaluated the effects of different temperatures (20, 27, and 33°C) and feeding rates (2.5% of biomass and apparent satiation) on digestive enzyme activity, gut histology, gut microbiota, growth-regulating gene (*IGF-I*), and stress-related gene (*HSP70*) in a 6-week dietary trial.

MATERIALS AND METHODS

Fish and experimental design

Juvenile Asian seabass (n=180, 85.0±3 g) was procured from Ramoz Company (Bushehr, Iran) and transferred to the laboratory of Marine Aquatic Research, Persian Gulf University (Bushehr, Iran). Before the main experiment commenced, the fish underwent a 2-week acclimatization period to laboratory-rearing conditions. During this period, the fish were fed twice daily with a commercial feed (Beyza, Iran) containing 47% crude protein, 17% crude fat, 2% crude fiber, and 14% ash. After this period, fish were distributed into 6 different treatments in triplicates (10 fish/300-L tank), including 3 different temperatures (20, 27, and 33°C) and 2 feeding rates (2.5% of biomass or ad libitum). The treatments were designed and named T1 (20°C, 2.5% biomass), T2 (20°C, apparent satiation), T3 (27°C, 2.5% biomass), T4 (27°C, apparent satiation), T5 (33°C, 2.5% biomass), and T6 (33°C, apparent satiation). Throughout the 6-week dietary trial, each tank received sand-filtered, dechlorinated, and UV-disinfected seawater, ensuring approximately 60-70% daily turnover of the rearing water. During the experiment, water physiochemical parameters, including salinity (48±2 ppt), pH (7.5±0.5), and dissolved oxygen (70-80% saturation), were monitored and adjusted if necessary. The photoperiod was artificially set as 12L: 12D.

The activity of digestive enzymes

To measure digestive enzyme activity, fish (9 fish per treatment) were anesthetized with (2-phenoxyethanol, (500 ppm, Afkhami *et al*., 2014; Zeynali *et al*., 2020), and then the whole gut was removed, washed twice with double distilled water, then homogenized in 100mM Tris-Hcl buffer containing 0.1 mM EDTA and 0.1% Triton X-100 (pH 7.8), and centrifuged at 30000×g for 12 min at 4 °C. The supernatant was then collected and kept frozen at -80°C (Furné *et al.*, 2008) until further analysis. A commercial lipase kit (Bionik, Canada) was used to measure the specific activity of lipase. The photometric measurement was based on the hydrolysis of 1,2-o-dilauryl-rac-3-glutaric acid-(6-methyresorufin) ester substrate, resulting in the production of 6-methyresorufin and glutaric acid-6-ethylresorufin ester.

Specific activity of amylase was measured using a commercial amylase kit (Bionik, Canada) based on 4, 6 ethylidene-(G7)-p-nitrophenyl-(G1)-alpha-D-maltoheptaside (Eps-G7) as substrate. The method described by (Anson, 1938) was used for measuring total protease activity using casein as the substrate. 1 mL of supernatant samples were added to a reaction mixture (1 mL of 1.5% azocasein solution, pH 7.0) and incubated for 10 min at 37°C. After that, 2 mL of 0.4 M trichloroacetic acid was added, the solution was filtered, and 2.5 mL of 0.4 M Na2CO3 and 0.5 mL of Folin reagent were added.

Samples were assessed for protein content using the Bradford method (Bradford, 1976) with bovine serum albumin as the standard (1 mg/mL). Subsequently, digestive enzyme activity was quantified as the change in absorbance per minute per milligram of soluble protein as follows:

Unit/mg protein= Abs (410nm)×1000×ml of reaction mixture/8800×mg protein in reaction mixture

Histological studies

At the end of the experiment, two fish (6 fish per treatment) were randomly selected from each tank for histological evaluations. Approximately 0.5 cm segments of the midgut were excised using a sterile scalpel and fixed in 10% formalin solution. Following established histological protocols, the gut samples underwent dehydration in a graded series of ethanol and xylene, followed by embedding in paraffin blocks. Then, 5µm transverse sections were provided, stained

with hematoxylin and eosin, and were assessed using light microscopy (Roberts, 2012). The images were processed for length and thickness values using ImageJ software.

Gut microbiota

In aseptic conditions, the intestine samples (9 fish per treatment) were washed homogenized in sterile saline solution (0.9% NaCl), and the homogenate was diluted as required. The fish's outer layer was disinfected using 70% ethanol before dissection. The suspensions were serially diluted (10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}) and 0.1 ml of the solution was spread onto TSA (Tryptic Soya Agar) plates. Total aerobic bacterial colonies were counted after incubation at 27°C for 48-72 hours (Rawling *et al.*, 2009).

Gene expression

The expression of genes of interest was evaluated in liver samples. A portion of liver tissue samples were removed, snap-frozen in liquid nitrogen, and stored at -80°C until further analysis. An RNA extraction kit (Cinnagen Iran) was used to extract the total RNA content of samples according to the manufacturer's instructions. The extracted RNA was quantified using a spectrophotometer (ND-1000, Nanodrop). The quality of extracted RNA was assessed on 1% agarose electrophoresis. The complementary DNA (cDNA) strand was subsequently synthesized using 1µg of total RNA and 10 pmol Random Hexamer primer and RevertAidTM First Strand cDNA Synthesis Kit (Fermentas, K1622). Specific primers (*HSP70*, *IGF-I*, and *Ef1a*) for real-time PCR analysis of Asian seabass were designed with Primer3Plus software (Table 1). The *Ef1a* gene served as the housekeeping gene. RT-PCR was performed using a real-time PCR machine (RotorGene, RG-2000, Sydney, Australia) in total volume of 12.5 µl containing 6.25 µl of 2X SYBR Green qPCR Master Mix (Cinnagen, Iran), 0.5 µl of cDNA, 0.5 µl of each primer, 0.1 µl Tag polymerase and 4.65 µl of double distilled and DNase free water (DEPC water). The amplification conditions were as follows: 95°C for 10 min, followed by 40 cycles at 95°C for 30 s, 60°C for 45 s and 72°C for 45. The average threshold cycle (Ct) was calculated for each sample and normalized to the housekeeping gene. The relative expression of genes was calculated based on the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Table 1. The sequences of the primers used in this experiment for *IGF-I* and *Hsp70* genes of Asian seabass (*Lates calcarifer*).

Gene name	Function	Sequences of primers	Accession number	Efficiency
IGF-I	Growth	F: ACGCTGCAGTTTGTATGTGG R: CCTTAGTCTTGGGAGGTGCA	XM_018697285.1	98%
Hsp70	Stress	F: AAGGCAGAGGATGATGTC R: TGCAGTCTGGTTCTTGTC	XM_018672747.1	94%
Ef1a	Housekeeping	F: AAATTGGCGGTATTGGAAC R: GGGAGCAAAGGTGACGAC	GQ507427.1	97%

Statistical analysis

The data were analyzed using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). Differences among the groups were evaluated through a two-way analysis of variance (ANOVA), followed by Tukey's multiple range post hoc test. Results are presented as means±standard error (n= 3), and statistical significance was determined at $P< 0.05$.

RESULTS

The activity of digestive enzymes

Table 2 showed no significant difference among treatments regarding total protease, amylase, and lipase enzyme activity (Table 2; $P> 0.05$). The interactive influence of temperature and feeding rate did not statistically affect the normal function of the digestive system. However, individuals from the T5 treatment exhibited the highest specific activities of lipase, amylase, and total protease, suggesting that elevated temperature (33°C) and a feeding rate of 2.5% biomass enhanced the activity of digestive enzymes.

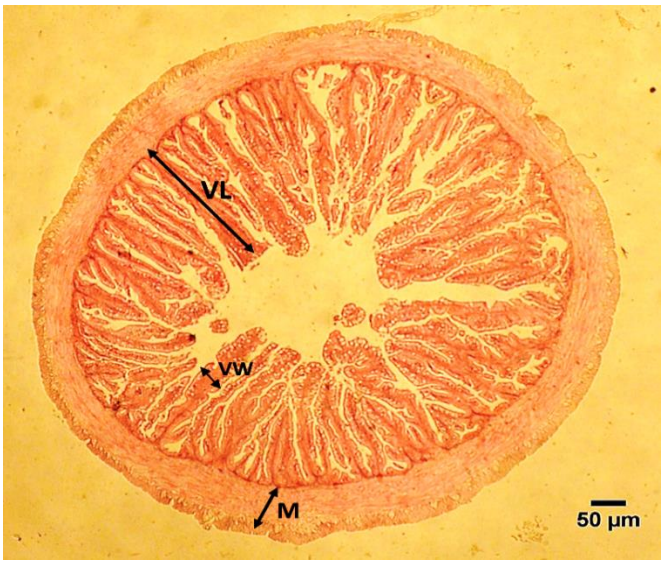
Table 2. The activity of digestive enzymes of Asian seabass (*Lates calcarifer*) reared at different temperatures and feeding rates for 6 weeks.

Treatment	Lipase (U/mg protein)	Amylase (U/mg protein)	Protease (U/mg protein)
T1	4.43±1.19	8.76±2.87	1.90±0.70
T2	4.36±0.15	7.76±2.17	1.90±0.34
T3	5.70±0.20	9.16±1.44	2.40±0.10
T4	4.93±1.78	8.56±1.10	2.20±0.10
T5	6.00±1.70	10.50±3.50	2.60±0.52
T6	4.25±0.05	7.40±1.00	2.23±0.35
	Two-way ANOVA		
Temperature	0.747	0.805	0.529
Feeding rate	0.675	0.811	0.181
Temperature × Feeding rate	0.095	.0244	0.235

T1 (20°C, 2.5% biomass), T2 (20°C, apparent satiation), T3 (27°C, 2.5% biomass), T4 (27°C, apparent satiation), T5 (33°C, 2.5% biomass), and T6 (33°C, apparent satiation). Data are presented as mean±SE. The absence of superscript letters indicates no significant difference ($P> 0.05$).

166 **Histological studies**

167 Histological studies were carried out to identify any significant changes in intestine tissue
168 morphology where muscularis thickness, villi height, and villi width were measured, as depicted
169 in Figure 1.



170

171 **Figure 1.** Details of intestinal histological structure of Asian seabass (*Lates calcarifer*) cultured
172 in different temperatures and feeding rates. VL: villi length; VW; villi width; M: muscular width
173 (H & E, 400X, Scale bar= 50 µm).
174
175 Results from histological evaluations are depicted in Table 3, which indicates no considerable
176 changes in muscular thickness, villi height, and width between different treatments (P> 0.05).
177 However, the results were complex and variable; thus, no definitive conclusions could be drawn
178 regarding the influence of temperature, feeding rate, or their combined effects on the measured
179 morphometric values of fish intestines.
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Table 3. Intestinal morphology of rainbow of Asian seabass (*Lates calcarifer*) reared at different temperatures and feeding rates for 6 weeks.

Treatment	*Muscularis thickness (µm)*	*Villi height (µm)*	*Villi width (µm)*
T1	44.08±6.38	160.20±16.19	84.46±1.96
T2	40.33±6.76	142.52±13.82	74.45±6.22
T3	47.96±8.99	135.67±84.56	62.73±3.66
T4	55.81±7.90	192.91±19.49	84.46±8.94
T5	55.70±9.70	159.75±11.28	74.14±4.55
T6	41.34±3.49	154.84±84.56	67.86±3.88
	Two-way ANOVA		
Temperature	0.778	0.095	0.054
Feeding rate	0.286	0.064	0.303
Temperature × Feeding rate	0.324	0.095	0.676

T1 (20°C, 2.5% biomass), T2 (20°C, apparent satiation), T3 (27°C, 2.5% biomass), T4 (27°C, apparent satiation), T5 (33°C, 2.5% biomass), and T6 (33°C, apparent satiation). Data represent mean±SD (n= 3). The absence of superscripts in each column indicates no significant difference (P> 0.05).

Gut microbiota

Figure 2 displays variations in bacterial counts in the intestines of fish subjected to different temperature and feeding rate conditions. The results indicate minor fluctuations in the total number of culturable bacteria across fish intestines; however, statistical analysis revealed no significant differences (P> 0.05). The highest bacteria were observed in T5, where fish were reared at high temperatures and fed 2.5% of biomass.

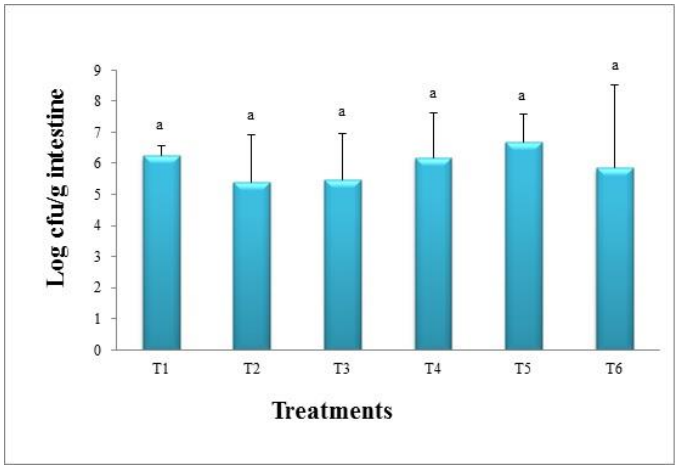

Figure 2. Bacterial colonies cultured from intestine tissue of Asian seabass (*Lates calcarifer*) after 6 weeks. T1 (20°C, apparent satiation), T2 (20°C, 2.5% biomass), T3 (27°C, apparent satiation), T4 (27°C, 2.5% biomass), T5 (33°C, apparent satiation), and T6 (33°C, 2.5% biomass). Data are presented as mean±SE. The same superscripts indicate no significant differences (P> 0.05).

Gene expression

The relative expression of *IGF-I* and *HSP70* genes was evaluated in fish reared under different temperatures and feeding rates, and the results are shown in Table 4. In contrast, temperature significantly affected the expression of *IGF-I* and *HSP70*, which is evident when comparing low-temperature treatments (T1 and T2) with other treatments. From the statistical point of view, the transcription levels of *IGF-I* and *HSP70* were the lowest in T1 and T2 ($P< 0.05$) and the highest in T5 and T6 in comparison with other treatments ($P< 0.05$).

Table 4. Relative expression of *IGF-I* and *HSP70* genes in liver tissue of Asia seabass *(Lates calcarifer*) reared under different temperatures and feeding rates for 6 weeks.

Treatment	IGF-I	HSP70
T1	1.00 ± 0.04^{b}	1.00 ± 0.24^{c}
T2	1.14 ± 0.24^{b}	1.13 ± 0.02^{c}
T3	3.27 ± 0.61^{a}	4.72 ± 0.44^{b}
T4	3.77 ± 0.24^{a}	4.62 ± 0.62^{b}
T5	3.56 ± 0.24^{a}	9.29 ± 1.69^{a}
T6	3.69 ± 0.76^{a}	10.20 ± 1.50^{a}
Two-way ANOVA		
Temperature	0.00	*0.00*
Feeding rate	0.354	*0.532*
Temperature × Feeding rate	0.723	*0.676*

T1 (20°C, 2.5% biomass), T2 (20°C, apparent satiation), T3 (27°C, 2.5% biomass), T4 (27°C, apparent satiation), T5 (33°C, 2.5% biomass), and T6 (33°C, apparent satiation). Data represent mean±SE (n= 3). Different superscripts in each column indicate significant differences ($P< 0.05$).

DISCUSSION

The current study investigated the interactive effects of different water temperatures and feeding rates on digestive enzyme activity, histology, and gut microbiota of Asian seabass. The present results revealed that different temperatures and feeding rates or their interaction (Two-way ANOVA analysis) do not significantly affect the activity of digestive enzymes and gut morphometrics. However, higher activity of digestive enzymes was found in individuals from T5 (33°C and 2.5% biomass) and T3 (27°C and 2.5% biomass), respectively. Furthermore, at molecular levels, significant changes were observed at the same water temperature but at different feeding rates. It is worth mentioning that no mortality was observed during the trial; however, 10-fold higher transcription levels of HSP-70 were

Other researchers have investigated similar hypotheses. For instance, Baloi et al. (2017) evaluated the effects of different feeding rates, ranging up to satiation levels, on juvenile

Brazilian sardine (*Sardinella brasiliensis*). Their study revealed a notable decline in total protease and amylase activities as feeding rates increased, while lipase activity demonstrated no significant variation in response to feeding rate adjustments. This is most likely associated with excessive food for the capacity of the target species' digestive system. It is also possible that a higher temperature will speed up metabolism and increase the digestive system's capacity; however, the present results did not support this (Table 2). In addition, Harpaz et al. (2005) observed elevated activity of brush border enzymes in Asian seabass under reduced feeding rates, coinciding with a decline in growth performance, which may be attributed to limited food availability. The discrepancy between results among studies might be attributed to the type of enzymes, various temperature ranges, tested feeding rate, duration of experiment, and fish species.

Volkoff and Rønnestad (2020) have suggested a direct effect of temperature on energy requirement. Temperature influences food intake, consumption, nutrient absorption, protein synthesis, and growth rate (Fauconneau, 1985). A temperature higher than optimal increases the gastrointestinal tract evacuation rate, which leaves less time for the digestion process and might reduce growth. In line with our results regarding the length and width of the villi and thickness of the intestinal muscular layer, Bowyer *et al.* (2012) have reported no significant histological changes.

Our findings suggest that rearing temperature and feeding rate do not significantly influence the number of cultivable bacteria in the fish guts across different treatments. Fish intestinal microbiota contributes to various physiological processes. Sugita *et al.* (1989) suggested that temperature can significantly affect bacterial populations in the water but not fish microflora. Liston (1957) has reported that in the intestine of skates (*Raja* spp) and lumen of Sole (*pleuvonectes microcephalus*), *Vibrio* spp. was dominant during all seasons. According to the literature, ecological and environmental factors can selectively promote the dominance of particular microbial populations (Ley *et al.*, 2008; Wong and Rawls, 2012). Fish can experience stress, reduced growth, and disruption in intestinal microbial communities at temperatures higher than optimal temperatures (Jobling, 1981). Moreover, bacterial growth in the fish intestine may

increase under elevated water temperatures (Huyben *et al.*, 2018). According to Hagi et al. (2004) a study involving three carp species and channel catfish revealed that lactic acid bacteria (LAB) populations in the fish gut showed no significant variation between summer temperatures (23–28°C) and winter temperatures (4–10°C). Soriano *et al.* (2018) have reported a notable alteration in gut microbiota in response to acclimation temperatures, while Huyben *et al.* (2018) documented dysbiosis in the intestinal microbiota in response to seasonal fluctuations. The interactive effects were also investigated at molecular levels, and as per the expression of *IGF-1* and *HSP70*, there were significant differences among different treatments. It is acknowledged that investigating *IGF-1* might help understand the correlations between temperature feeding rate and fish growth. The present study aligns with reports from rainbow trout (Chauvigné *et al.*, 2003) and chinook salmon (Beckman *et al.*, 1998), which experienced an increase in *IGF-1* correlated with the increased water temperature. Since fish did not experience any growth retardation, we suggest that the increased *HSP70* may not have occurred due to stress-mediated protein damage but rather as enhanced cytoprotection (Deane and Woo, 2005) which requires further research. *HSP70* gene expression was affected in different groups. Research has demonstrated that small heat shock proteins play a role in responding to temperature fluctuations (Podrabsky and Somero, 2004). Additionally, studies have reported that chronic acclimation of silver sea bream (*Sparus sarba*) to cold temperatures (12°C) led to an upregulation of *HSP70* compared to warmer temperatures (25°C) (Deane and Woo, 2005). This phenomenon may be attributed to stress-induced protein damage, as documented in prior studies (Ananthan *et al.*, 1986) and increased levels of *HSP70* have been associated with enhanced cytoprotection and prevention of cell apoptosis (Deane and Woo, 2005).

CONCLUSIONS

The present findings suggest that variations in temperatures and feeding rates do not significantly impact digestive enzymes, intestinal histology, and gut microbiota of Asian seabass over 6 weeks. However, temperature and feeding rate did influence the transcription of genes related to growth and stress responses, such as *IGF-I* and *HSP70*. These results underscore the need for further

investigation into how different temperatures and feeding rates affect the immune response and antioxidant capacity of Asian seabass at the molecular level.

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