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

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7 ABSTRACT

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The current study evaluated the interactive impacts of water temperature and feeding rate on 8 digestive enzymes, intestine histology, growth and stress-related genes, and cultivable intestinal 9 microbiota of Asian seabass (Lates calcarifer). For this purpose, 180 fish (85.0±3.0 g) were 10 reared at three different temperatures (20, 27, and 33°C) and two feeding rates (apparent satiation 11 and 2.5% of biomass) with three replications for 6 weeks. The results revealed no significant 12 differences among different treatments regarding the activity of digestive enzymes (P > 0.05) of 13 fish reared under different temperatures and feeding rates. The length, width, and thickness of 14 intestinal villi were unaffected by different temperatures and feeding rates (P>0.05). In addition, 15 no variations were found in the total aerobic bacterial count of fish gut from different 16 experimental groups (P> 0.05). At the molecular level, IGF-I and HSP70 coding genes were 17 found to be highly expressed in experimental treatments (P < 0.05). To conclude, the present 18 study showed that temperatures between 27 to 33°C are more optimal for Asian seabass, and the 19 different temperatures and feeding rates do not affect digestive enzymes, intestine histology, and 20 gut microbiota after 6 weeks. 21

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

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28 INTRODUCTION

Fish live in a 3-dimensional complex aquatic environment, and since fish are poikilothermic 29 animals, surrounding temperature is a major factor that regulates fish's metabolism and growth 30 rate (Frv. 1971: Groot et al., 1996). Fish are believed to perform best at their optimum 31 temperature, which varies even for different life stages of one species (Pedersen and Jobling, 32 1989), where feed utilization efficiency is maximum. Thus, for the best nutrition management, 33 aquafeed manufacturing companies often provide a range of feeding rates according to water 34 temperature for each specific growing stage of the target species. Therefore, knowing the 35 optimum temperature for each species can help with efficient production and increase the overall 36 yield. It is important to mention that feed accounts for 50 to 80% of the total aquaculture 37 production costs. Moreover, underfeeding or malnourishment might render fish more susceptible 38 to diseases and result in mass mortalities, while overfeeding results in feed loss and water quality 39 deterioration, making aquaculture practice less profitable. 40

The size of the fish and water temperature are important factors for optimizing feeding rates 41 (Kestemont and Baras, 2001; Wang, Xu, and Kestemont, 2009). For instance, the best growth 42 performance of pike perch (Sander lucioperca) was obtained when fish were fed to apparent 43 satiation (Ming et al., 2013). Given that, the rearing temperature and feeding rate are closely 44 interlinked and affect various physiological processes of fish. The impacts of feeding rate and 45 different rearing temperatures on various physiological aspects of fish have been reported by 46 other researchers (Fang et al., 2010; Kim et al., 2014; Baloi et al., 2017; Volkoff and Rønnestad, 47 2020). Both the feeding rate and the feeding time greatly influenced the activity of digestive 48 enzymes. Moreover, several factors, such as enough enzyme levels and time for digestion and 49 absorption, can affect the digestion process (Harpaz et al., 2005). Furthermore, water temperature 50 can affect digestive enzyme activities by affecting evacuation time (Temming and Herrmann, 51 2001). 52

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 56 potential when fed below or at an apparent satiation level, depending on the species (Han et al., 57 2004; Fang *et al.*, 2010). In addition, the increase in temperature can impact the digestive system, 58 intestinal microbiota, and, consequently, growth performance. However, the combined effects of 59 rearing temperature and feeding rate on have not been well studied in Asian seabass. Thus, in this 60 study, we evaluated the effects of different temperatures (20, 27, and 33°C) and feeding rates 61 (2.5% of biomass and apparent satiation) on digestive enzyme activity, gut histology, gut 62 microbiota, growth-regulating gene (IGF-I), and stress-related gene (HSP70) in a 6-week dietary 63 trial. 64

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MATERIALS AND METHODS 66

Fish and experimental design 67

Juvenile Asian seabass (n=180, 85.0±3 g) was procured from Ramoz Company (Bushehr, Iran) 68 and transferred to the laboratory of Marine Aquatic Research, Persian Gulf University (Bushehr, 69 Iran). Before the main experiment commenced, the fish underwent a 2-week acclimatization 70 period to laboratory-rearing conditions. During this period, the fish were fed twice daily with a 71 commercial feed (Beyza, Iran) containing 47% crude protein, 17% crude fat, 2% crude fiber, and 72 73 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%) 74 of biomass or ad libitum). The treatments were designed and named T1 (20°C, 2.5% biomass), 75 T2 (20°C, apparent satiation), T3 (27°C, 2.5% biomass), T4 (27°C, apparent satiation), T5 (33°C, 76 2.5% biomass), and T6 (33°C, apparent satiation). Throughout the 6-week dietary trial, each tank 77 received sand-filtered, dechlorinated, and UV-disinfected seawater, ensuring approximately 60-78 70% daily turnover of the rearing water. During the experiment, water physiochemical 79 parameters, including salinity (48 ± 2 ppt), pH (7.5 ± 0.5), and dissolved oxygen (70-80%80 saturation), were monitored and adjusted if necessary. The photoperiod was artificially set as 81 82 12L: 12D.

85 The activity of digestive enzymes

- 86 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 87 was removed, washed twice with double distilled water, then homogenized in 100mM Tris-Hcl 88 buffer containing 0.1 mM EDTA and 0.1% Triton X-100 (pH 7.8), and centrifuged at 30000×g 89 for 12 min at 4 °C. The supernatant was then collected and kept frozen at -80°C (Furné et al., 90 2008) until further analysis. A commercial lipase kit (Bionik, Canada) was used to measure the 91 specific activity of lipase. The photometric measurement was based on the hydrolysis of 1,2-o-92 dilauryl-rac-3-glutaric acid-(6-methyresorufin) ester substrate, resulting in the production of 6-93 methyresorufin and glutaric acid-6-ethylresorufin ester. 94 Specific activity of amylase was measured using a commercial amylase kit (Bionik, Canada) 95
- 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:
- 105 Unit/mg protein= Abs (410nm)×1000×ml of reaction mixture/8800×mg protein in reaction
 106 mixture
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108 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.

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117 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).

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125 Gene expression

The expression of genes of interest was evaluated in liver samples. A portion of liver tissue 126 samples were removed, snap-frozen in liquid nitrogen, and stored at -80°C until further analysis. 127 An RNA extraction kit (Cinnagen Iran) was used to extract the total RNA content of samples 128 according to the manufacturer's instructions. The extracted RNA was quantified using a 129 spectrophotometer (ND-1000, Nanodrop). The quality of extracted RNA was assessed on 1% 130 agarose electrophoresis. The complementary DNA (cDNA) strand was subsequently synthesized 131 using 1µg of total RNA and 10 pmol Random Hexamer primer and RevertAidTM First Strand 132 cDNA Synthesis Kit (Fermentas, K1622). Specific primers (HSP70, IGF-I, and Ef1a) for real-133 time PCR analysis of Asian seabass were designed with Primer3Plus software (Table 1). The 134 Efla gene served as the housekeeping gene. RT-PCR was performed using a real-time PCR 135 machine (RotorGene, RG-2000, Sydney, Australia) in total volume of 12.5 µl containing 6.25 µl 136 of 2X SYBR Green qPCR Master Mix (Cinnagen, Iran), 0.5 µl of cDNA, 0.5 µl of each primer, 137 138 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 30 139 140 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 141 calculated based on the $2^{-\Delta \Delta Ct}$ method (Livak and Schmittgen, 2001). 142

Table 1. The sequences of the primers used in this experiment for IGF-I and Hsp70 genes of 143 Asian seabass (Lates calcarifer). 144

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

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Statistical analysis 146

The data were analyzed using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). Differences 147

among the groups were evaluated through a two-way analysis of variance (ANOVA), followed 148

by Tukey's multiple range post hoc test. Results are presented as means \pm standard error (n= 3), 149

and statistical significance was determined at P < 0.05. 150

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152 RESULTS

The activity of digestive enzymes 153

154 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 155 rate did not statistically affect the normal function of the digestive system. However, individuals 156 from the T5 treatment exhibited the highest specific activities of lipase, amylase, and total 157 158 protease, suggesting that elevated temperature (33°C) and a feeding rate of 2.5% biomass enhanced the activity of digestive enzymes. 159

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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		
Τ2	4.36±0.15	7.76±2.17	1.90±0.34		
Т3	5.70±0.20	9.16±1.44	2.40±0.10		
Τ4	4.93±1.78	8.56 ± 1.10	2.20±0.10		
Т5	6.00±1.70	10.50 ± 3.50	2.60±0.52		
Тб	4.25±0.05	$7.40{\pm}1.00$	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 \pm 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
- in Figure 1.



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Figure 1. Details of intestinal histological structure of Asian seabass (*Lates calcarifer*) cultured
in different temperatures and feeding rates. VL: villi length; VW; villi width; M: muscular width
(H & E, 400X, Scale bar= 50 μm).

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Results from histological evaluations are depicted in Table 3, which indicates no considerable
changes in muscular thickness, villi height, and width between different treatments (P> 0.05).
However, the results were complex and variable; thus, no definitive conclusions could be drawn
regarding the influence of temperature, feeding rate, or their combined effects on the measured
morphometric values of fish intestines.

185	Table3.	Intestinal	morphology	of	rainbow	of	Asian	seabass	(Lates
186	<i>calcarifer</i>)) reared at d	lifferent tempe	erati	ares and fe	edi	ng rates	for 6 wee	eks.

culculifier) realed at different temperatures and reculing fates for 0 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			
<i>T3</i>	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			
<i>T5</i>	55.70±9.70	159.75±11.28	74.14±4.55			
<i>T6</i>	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), 7	Γ2 (20°C, apparent satiatio	n), T3 (27°C, 2.5	5% biomass), T4			
(27°C, apparent satiation), 7	15 (33°C, 2.5% biomass), a	and T6 (33°C, ap	parent satiation).			
Data represent mean±SD (n=	= 3). The absence of supers	cripts in each colu	umn indicates no			

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192 Gut microbiota

significant difference (P > 0.05).

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.



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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 \pm SE. The same superscripts indicate no significant differences (P> 0.05).

205 Gene expression

206 The relative expression of *IGF-I* and *HSP70* genes was evaluated in fish reared under different

207 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).

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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.

v /	0	
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}
Т3	3.27±0.61ª	$4.72 {\pm} 0.44^{b}$
T4	3.77±0.24 ^a	4.62 ± 0.62^{b}
Т5	3.56±0.24 ^a	$9.29{\pm}1.69^{a}$
T6	3.69 ± 0.76^{a}	10.20 ± 1.50^{a}
Two-way A	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).

219 **DISCUSSION**

220 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 221 results revealed that different temperatures and feeding rates or their interaction (Two-way 222 ANOVA analysis) do not significantly affect the activity of digestive enzymes and gut 223 morphometrics. However, higher activity of digestive enzymes was found in individuals from T5 224 (33°C and 2.5% biomass) and T3 (27°C and 2.5% biomass), respectively. Furthermore, at 225 molecular levels, significant changes were observed at the same water temperature but at 226 different feeding rates. It is worth mentioning that no mortality was observed during the trial; 227 however, 10-fold higher transcription levels of HSP-70 were 228

229 Other researchers have investigated similar hypotheses. For instance, Baloi et al. (2017) 230 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 231 and amylase activities as feeding rates increased, while lipase activity demonstrated no 232 significant variation in response to feeding rate adjustments. This is most likely associated with 233 excessive food for the capacity of the target species' digestive system. It is also possible that a 234 higher temperature will speed up metabolism and increase the digestive system's capacity; 235 236 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, 237 coinciding with a decline in growth performance, which may be attributed to limited food 238 availability. The discrepancy between results among studies might be attributed to the type of 239 enzymes, various temperature ranges, tested feeding rate, duration of experiment, and fish 240 species. 241

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.

249 Our findings suggest that rearing temperature and feeding rate do not significantly influence the 250 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 251 252 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 253 (pleuvonectes microcephalus), Vibrio spp. was dominant during all seasons. According to the 254 literature, ecological and environmental factors can selectively promote the dominance of 255 particular microbial populations (Ley et al., 2008; Wong and Rawls, 2012). Fish can experience 256 stress, reduced growth, and disruption in intestinal microbial communities at temperatures higher 257 than optimal temperatures (Jobling, 1981). Moreover, bacterial growth in the fish intestine may 258

increase under elevated water temperatures (Huyben et al., 2018). According to Hagi et al. 259 (2004) a study involving three carp species and channel catfish revealed that lactic acid bacteria 260 (LAB) populations in the fish gut showed no significant variation between summer temperatures 261 (23-28°C) and winter temperatures (4-10°C). Soriano et al. (2018) have reported a notable 262 alteration in gut microbiota in response to acclimation temperatures, while Huyben et al. (2018) 263 documented dysbiosis in the intestinal microbiota in response to seasonal fluctuations. The 264 interactive effects were also investigated at molecular levels, and as per the expression of IGF-1 265 and HSP70, there were significant differences among different treatments. It is acknowledged 266 that investigating *IGF-1* might help understand the correlations between temperature feeding rate 267 and fish growth. The present study aligns with reports from rainbow trout (Chauvigné et al., 268 2003) and chinook salmon (Beckman et al., 1998), which experienced an increase in IGF-1 269 correlated with the increased water temperature. Since fish did not experience any growth 270 retardation, we suggest that the increased HSP70 may not have occurred due to stress-mediated 271 protein damage but rather as enhanced cytoprotection (Deane and Woo, 2005) which requires 272 further research. HSP70 gene expression was affected in different groups. Research has 273 demonstrated that small heat shock proteins play a role in responding to temperature fluctuations 274 (Podrabsky and Somero, 2004). Additionally, studies have reported that chronic acclimation of 275 silver sea bream (Sparus sarba) to cold temperatures (12°C) led to an upregulation of HSP70 276 277 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., 278 1986) and increased levels of HSP70 have been associated with enhanced cytoprotection and 279 prevention of cell apoptosis (Deane and Woo, 2005). 280

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282 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 andantioxidant capacity of Asian seabass at the molecular level.
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