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Effects of temperature and diet on pepsin enzyme activity of TGGG hybrid grouper, *Epinephelus fuscoguttatus* ♀ x *E. lanceolatus* ♂

M. De[1], M.A. Ghaffar[3], Y. Bakar[1], Z.C. Cob[1], S.K. Mazumder[4] and S.K. Das[1,2]*

[1]Department of Earth Sciences and Environment, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, Bangi, 43600, Malaysia
[2]Marine Ecosystem Research Centre (EKOMAR), Faculty of Science and Technology, Universiti Kebangsaan Malaysia, Bangi, 43600, Malaysia
[3]Institute of Marine Biotechnology, Universiti Malaysia Terengganu, Kuala Terengganu, 21300, Malaysia
[4]Department of Genetics and Fish Breeding, Faculty of Fisheries, Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur, 1706, Bangladesh

***Corresponding Author Email :** simon@ukm.edu.my

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Abstract

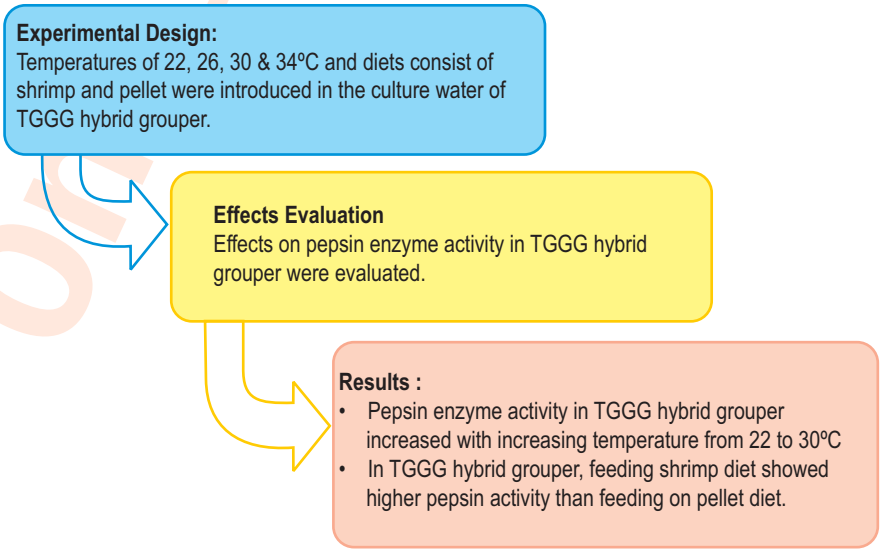
Aim: This study explores the influence of temperature and diet on pepsin enzyme activity in TGGG hybrid groupers.

Methodology: Two hundred TGGG groupers (body weight 200±10 g; total length 22.5±1 cm) were equally distributed in three rearing tanks (1500 l capacity). After three weeks of acclimatization, the groupers were kept in 40 tanks (5 fish per tank, 20 tanks with pelleted diet and 20 with shrimp diets) for 30 days. All treatments were replicated five times.

Results: Pepsin activity increased with increased temperature, from 22°C (2.10 U mg protein^{-1}) to 30°C (5.64 U mg protein^{-1}) when the groupers were given either pellet or shrimp as diet. The shrimp-fed group showed significantly increased pepsin activity compared to pellet-fed fish.

Interpretation: The pepsin enzyme activities in TGGG hybrid groupers were significantly influenced by temperature and diet, but the interaction of these two factors was insignificant. The results suggest that shrimp-diets given at 30°C water was ideal for rearing TGGG hybrid groupers. This suggests that combination proliferates enzymatic activity, which may lead to faster digestion and faster growth rates compared to other combinations.

Key words: Digestive enzymes, Diet, *Epinephelus fuscoguttatus*, Hybrid grouper, Temperature



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Introduction

Very little information is available regarding the role of temperature and food in stimulating the secretion of digestive enzymes in fish. A better understanding of digestive enzymes leads to a better understanding of the overall digestive mechanisms in fish and how these organisms adapt to changes in the nutritional environment (Uys and Hecht, 1987). The digestive enzyme activities of fish are associated with innate feeding habits and diet composition (Ray, 1988). Carnivorous fishes naturally represent high protease activities, while omnivorous and herbivorous fishes exhibit active carbohydrate activities (Ugolev and Kuz'mina, 1994).

Several factors age, size, water temperature, pH and feeding regimes) influence digestive enzyme activities in fishes (Gildberg *et al*., 1990; Tanji *et al*., 2007; Klomklao *et al*., 2007; Brier *et al*., 2007; Kuz'mina, 1996), but research on the combined effect of temperature and diet on fish enzyme activities has been rarely reported. The only currently available report on the subject was reported by Mazumder *et al*. (2018) on the Malabar blood snapper (*Lutjanus malabaricus*).

TGGG hybrid grouper (*Epinephelus fuscoguttatus* ♀ × *Epinephelus lanceolatus* ♂) is a carnivorous fish (Othman *et al*., 2015; De *et al*., 2016a). Under farming conditions, the TGGG hybrid grouper accepts all types of processed feeds and trash fish, and exhibit good growth performance and resiliency (Ch'ng and Senoo, 2008; De *et al*., 2016a, b). There is an urgent need to better understand enzymatic function and to determine the nutritional requirements of TGGG hybrid groupers by analyzing digestive enzyme assays. Digestive enzyme assays of TGGG hybrid groupers have yet to be fully explored, despite the existence of some biological studies focused on sexual development, growth, blood analyses, feeding performance, gastric emptying, and stress levels (Luin *et al*., 2013; Jiang *et al*., 2015; Othman *et al*., 2015; Noor *et al*., 2018, 2019a, 2019b; Das *et al*. 2021; De *et al*., 2016a, b, 2019). This research focus on the levels of pepsin enzyme activity under different temperature and diet conditions in TGGG hybrid groupers.

Materials and Methods

Experimental setup: Two hundred TGGG hybrid groupers (body weight 200±10 g; total length 22.5±1 cm) were obtained from a fish farm in Selangor, Malaysia (2°49'0" N,101°30'0" E), then transported to the wet laboratory, at the Faculty of Science and Technology, UKM. The fish were stocked in 3 tanks, each of which had a capacity of 1500 l. The grouper samples were acclimatized for 3 weeks, after which they were allocated to 40 tanks with 5 fish per tank. Twenty tanks were fed pelleted diet and the other 20 tanks were fed shrimp diet. The tanks measured 0.6 m^2 and 0.58 m (high). A thermostat (Dophin 150 W, Malaysia) and chiller (Arctica R22, Malaysia) were used to obtain the desired experimental temperatures (22, 26, 30 and 34°C).The fish were fed twice daily with pellets (crude protein 46%; Leong Hup brand) or freshly thawed shrimp (crude protein 58%; *Acetes* sp.) for 30 days. The experiments were carried out in five replicates.

Tissue preparation: After 30 days, the TGGG hybrid groupers were starved for 48 hr to empty their stomachs. They were then anesthetized to measure body weight and total length. An incision was made in the esophagus and cloaca of the fish and their entire gut was removed. Ice-cold saline phosphate buffer (pH 7.4) was used to wash the stomach, then preserve it at -80°C for further analyses.

Homogenization: Prior to homogenization, the stomachs were carefully thawed. Homogenization was performed using a homogenizer (Dyna Stream BT200, China) in which the stomach tissue and buffer (50 mM Tris-HCl buffer solution with pH 7.4) were diluted in 1:20 ratio, and centrifuged at 10,000 rpm at 4 ºC for 15 min (Thermo Fischer Scientific BIOS 16, USA). The supernatant was then collected at -20°C and stored until used in enzyme assays.

Enzyme assays: The assays were measured for their absorbance using a spectrophotometer (Thermo Fisher Scientific GENESYS™Spectrophotometer 10S, USA). Each assay was compared to the homogenate and substrate blanks. An assay kit from BioRad® was utilized to calculate the total protein content in the supernatant (Bradford, 1976).

Estimation of crude protein: A standard curve against the blank was plotted to determine the total protein concentration in each assay. The protein content and enzyme assays were estimated following the standard procedure of Bradford (1976).

Pepsin assay: Extracted crude enzymes (100 µl) were added to 500 µl of substrate (2 % bovine hemoglobin). Blank tubes consisting of 500 µl substrate were also prepared. The tubes were left to incubate for 10 m at 37°C. To stop the process, 5% TCA was added to each test tube, except for the blank tubes, which contained 100 µl crude enzyme extract was mixed in the blank tube to stop the reaction process. The tubes were then allowed to settle for 5 min at room temperature. Later, the tubes were centrifuged for 5 min at 12000 × g. Finally, specific enzyme activity (U mg protein^{-1}) was quantified using absorbance readings at 280 nm.

Statistical analyses: Tests for normality using Kolmogorov-Smirnov and Bartlett's homogeneity of variance for different groups were done prior to further statistical analyses (Sokal and Rohlf, 1995). Specific pepsin enzyme activities were then analyzed by Two-way ANOVA. A pairwise *post-hoc* Tukey test was run to find significant differences at $p<0.05$. For pepsin activity, a non-linear polynomial cubic model was fitted to determine the significance of different temperatures and diets.

Statistical software used included MicroCalc. Origin™ Ver. 9.0 (OriginLab, Northampton, MA) and MINITAB ver. 20 (StatSoft Inc., USA).

Results and Discussion

The ANOVA result indicated that the temperature–diet interaction was insignificant ($p>0.05$) and did not affect the interpretation of main effects. Pepsin enzyme activities in TGGG hybrid groupers were significantly influenced by different temperatures and diets (Table 1).

Tukey tests showed that the mean pepsin activity differed significantly at four experimental temperatures. The highest mean pepsin activity (5.64 U mg protein^{-1}) was observed at 30ºC, while the lowest activity (2.10 U mg protein^{-1}) was observed at 22ºC. As the temperature increased, pepsin activity also increased for both diets up to 30°C. A further increase in temperature to 34°C resulted in decreased pepsin activity (3.37 U mg protein^{-1}). The shrimp-fed group showed significantly higher pepsin activity (4.39 U mg protein^{-1}) than the pellet-fed group (3.52 U mg protein^{-1}).

The relationship between specific pepsin activity and temperature incorporated with the polynomial cubic model for the pellet diet is presented in Fig. 1, where the data aligned well with the dataset (r^2=0.99). The results indicate that 30°C is an ideal temperature for pepsin enzyme activity in TGGG hybrid groupers fed either pellets or shrimp; however, Muyan *et al*. (2006) reported that the maximum pepsin enzyme activity in turbot *Scophthalmus maximus* occurred between 30°C to 60°C. In this study, the lowest and highest activities were observed within 8°C range; however, enzyme activity was halved with only 5°C temperature change in turbot (*S. maximus;* Muyan *et al*., 2006) and red fish

Table 1 : Two-way ANOVA on pepsin activity in TGGG hybrid grouper

Source	DF	SS	MS	F
Temperature (°C)	3	71.89	23.96	71.76*
Diet	1	7.73	7.73	23.16*
Temp × Diet	3	0.6302	0.2101	0.63
Error	32	10.6858	0.3339	
Total	39	90.9412		

*: $p<0.05$, DF: Degrees of Freedom, SS: some of squares, MS: Mean square, F: F-test

(*Sebastes mentella*; Munilla-Morán and Saborido-Rey, 1996). It has been suggested that the increase in enzyme activity with temperature increase is due to frequent collision of substrates with active sites (Reece *et al*., 2011).

Pepsin enzyme activity was higher at all temperatures when combined with shrimp diet (shrimp comprises more protein, 58%) as compared to pellet diet (protein level 50%), which is unsurprising, since groupers are carnivorous fish. This is consistent with the study of Kamarudin *et al*. (2013) who focused on other tropical fishes. It has been reported that the type of diet directly affects the enzyme activity in fish (Hardewig and van Dijk, 2003) and can be used to identify particular part of a fish's diet (van der Veer, 1986). The increased enzymatic activity in our study positively influenced growth, which concurs with the results of Mazumder *et al*. (2018) in Malabar blood snapper (*L. malabaricus*) and Das *et al*. (2018) mahseer (*Tor tambroides*).

In general, pepsin enzymes digest the protein in the stomach and determine when it passes to the intestine, where it is further degraded into smaller peptides and free amino acids by alkaline proteases (Hardewig and van Dijk, 2003). It has been

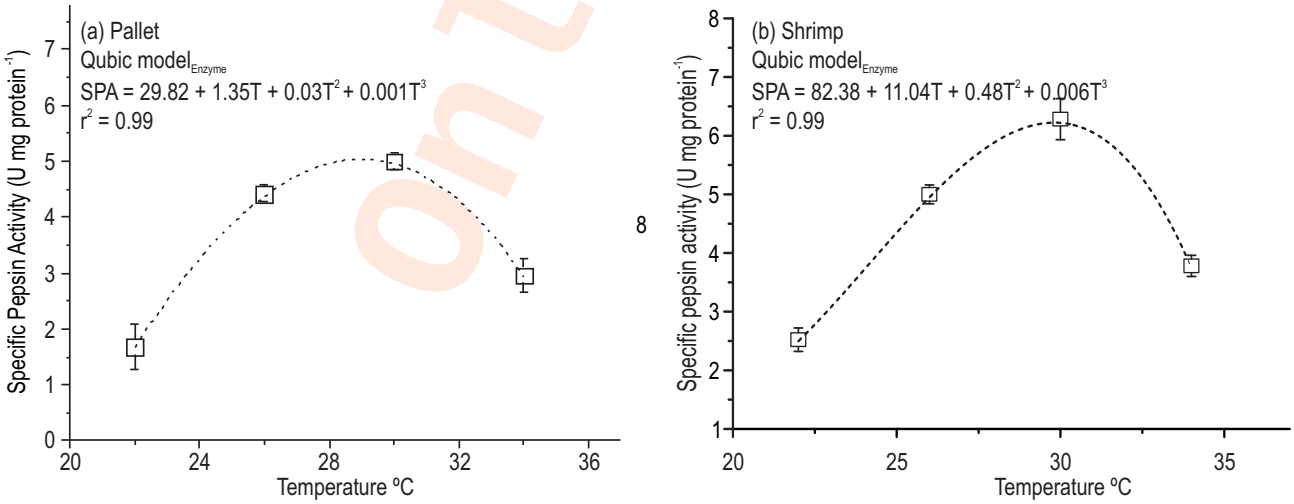

Fig. 2 : Relationship between specific activity of pepsin enzyme of TGGG hybrid grouper at different temperatures and diets: (a) pellet diet and (b) shrimp diet. Values are means ± S.E., SPA: Specific pepsin activity, dotted line denotes enzyme activity.

suggested that TGGG hybrid groupers like other carnivorous fishes followed and comply similar digestion mechanism with the active association of pepsin enzymes. Thus, pepsin in the stomach is vital for protein digestion in carnivorous fishes and aid in the breakdown of large-chain polypeptides (Tengjaroenkul *et al*., 2000).

The activity of pepsin enzyme was strongly dependent on temperature (Fig. 2). Similar observations were recorded among other marine fishes, namely rainbow trout (*Salmo gairdneri*; Fauconneau *et al*., 1983) and Malabar blood snapper (*Lutjanus malabaricus*; Mazumder *et al*., 2018). Temperature affects fish digestion, as observed in the TGGG hybrid grouper (De *et al*., 2016a) and other fishes, Roach (*Rutilus rutilus*; Hofer, 1979, 1982; Smith, 1975) and Malabar blood snapper (*Lutjjanus malabaricus*; Mazumder *et al*., 2018). Pepsin enzyme activity was optimized at 30ºC, which is higher than their common habitat temperature of 28ºC in coastal waters where TGGG hybrid groupers are reared. This corresponds to the rising temperature trend in other marine fishes, such as red drum (*Sciaenops ocellatus*; Lazo *et al*., 2007).

We advocate the culture of TGGG hybrid groupers at 30°C in combination with a shrimp diet. Enzymatic activity thrives at 30°C and leads to faster digestion process at potentially faster growth rate. The results of this study can be used as a basis for enhancing the management approach for TGGG hybrid groupers or any other Malaysian or tropical grouper species. Moreover, the results obtained from this study may be used in food management to optimize the aquaculture production of this hybrid grouper.

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Add-on Information

Authors' contribution : M.De: Experimentation, data analysis and writing-original draft; **M.A. Ghaffar:** Project administration; **Y. Bakar:** Statistical analysis; **Z.C. Cob:** Resource administration; **S.K. Mazumder:** Data analysis; **S.K. Das:** Conceptualization, data analysis, review, editing and finalizing the manuscript.

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Ethical approval : Not Applicable

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