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Evaluation of the optimal temperature for digestive protease function in the hybrid catfish *Clarias gariepinus* × *Heterobranchus longifilis*

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SUMMARY

(1) Background: Water temperature is a key determinant of fish digestion. In vitro methods provide a reliable approach to assess enzyme activity, which is especially important in carnivorous catfish with protein-rich diets. Although the hybrid catfish Clarias gariepinus × Heterobranchus longifilis is widely farmed in Hungary, its optimal temperature for maximizing digestibility remains under investigation.; (2) Methods: Fish were reared at 23 ºC, 25 ºC, and 27 ºC, and stomach and proximal intestine content samples were collected, and it was determined the protease activity (3) Results: No statistically significant differences were observed in enzyme efficiency, crude protease activity (p > 0.05). Despite not significant differences, the stomach and intestinal, protease activity tended to be higher at 25 °C (3.41 ± 0.16 U/mL), and, while in the intestines was higher at 27 °C (2.03 ± 0.27 U/mL). Stomach activity was higher at 27 ºC, at 27 °C (2.03 ± 0.27 U/mL, respectively. (4) Conclusions: Stomach protease activity tended to be higher at 25 °C, while intestinal activity was highest at 25–27 °C. These results may indicate a better starting digestion that supports feed digestion.

Keywords: *in vitro; fish digestion; protease activity; temperature*

INTRODUCTION

Fishes are adapted to diverse environments with unique characteristics. A good quality water sources is key to shape fish physiology, metabolism, growth, survival, and behaviour. The most important water parameters include physical factors such as temperature, turbidity, and salinity; chemical factors like dissolved oxygen, pH, and inorganic nitrogen; and contaminants including microplastics and crude oil (Zhang et al., 2025). The optimal water temperature for fish is crucial for physiology, growth, and behaviour. Studies on Atlantic salmon, brown trout, and Arctic charr, temperatures higher than 3 ºC, led to reduced growth (Elliott and Elliot, 2010). Hybrid striped bass exhibit lower leukocyte counts at 10 °C and increased protein, albumin, and chloride at 29 °C (Hrubec at al., 1997), while red sea bream showed elevated glucose, AST, and cortisol at 7 °C along with decreased protein and potassium (Hwang et al., 2012). Multiple-species investigations indicate that fish facing temperatures above their preferred ranges experience increased mortality and stress—70% of such cases—in addition to behavioural impairments like reduced swimming efficiency and heightened predation risk (Gale et al., 2013).

Most of the research studies related with water temperature in catfish sp. are mainly focused on growth and feed efficiency. Optimal water temperatures for growth and feed efficiency in catfish generally fall between 27 ℃ and 30 ℃. For example, studies on channel catfish consistently report highest performance—measured by growth rate and feed conversion—within this range, while yellow and European catfish also achieve peak growth at approximately 27 ℃ (Zhang et al., 2017; Hilge et al., 1985; Suja et al., 2009; Buentello et al., 2000). Chinese catfish juveniles grow fastest at 25 ℃, although survival improves at 30 ℃ (Anderson and Fast, 1991). Butter catfish exhibit superior hematological and growth outcomes at 30 ℃ (Islam et al., 2022), and broadhead catfish shows fastest larval growth at 30 ℃ with comparable survival between 27 ℃ and 35 ℃ (Mollah, 1984). In transgenic Mutiara catfish, reproductive parameters such as testosterone levels and spermatozoa density peak at 24 ℃, with benefits extending to 28 ℃ (Buwono, 2024). Thus, studies indicate that a water temperature within the 27 ℃–30 ℃ range most often produces optimal growth and feed utilization, with species-specific variations noted for survival and reproductive outcomes.

The hybrid catfish (*Clarias gariepinus* × *Heterobranchus longifilis*) is widely farmed in Hungary due to its desirable traits, including the hardiness of *C. gariepinus*, the fast growth of both *C. gariepinus* and *H. longifilis*, the high flesh yield of *H. longifilis*, and its delayed sexual maturation, which makes it ideal for reaching market weights of up to 6 kilograms. Despite its economic significance, there is limited knowledge about the optimal water temperature for maximizing digestive enzyme activity in this hybrid species. This study aimed to determine the optimal temperature for protease activity. Enzyme activity was assessed *in vitro* at three different water temperatures—23 °C, 25 °C, and 27 °C—to identify the temperature that supports the highest proteolytic performance.

MATERIALS AND METHODS

Ethical statement

The animal experiment was authorized by the Hajdú-Bihar County Government Office (project evaluation No. KA-4471), following the approval of the University of Debrecen Animal Welfare Committee

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and the Scientific Ethical Council on Animal Experimentation (approval date: 26 August 2025). All procedures were conducted in compliance with Act XXVIII of 1998 on the Protection and Humane Treatment of Animals and Government Decree No. 40/2013 (II.14.) on animal experiments.

Experimental fish

Three- to six-month-old hybrid African catfish (*Clarias gariepinus* × *Heterobranchus longifilis*) with an average body weight of 200 to 400 g were sourced from the fish laboratory at the University of Debrecen. The fish were maintained in a 1000-litre tank within a recirculated water system for two months. They were fed 5% of their body weight daily. Before sampling, the fish were fed to ensure their stomachs and intestines were full, activating gastric enzymes for subsequent collection and standardisation. Fish were sampled from three different water temperatures: 23 °C, 25 °C, and 27 °C. From each temperature group, six fish were collected for analysis.

Experimental feed

Hybrid catfish *Clarias gariepinus* × *Heterobranchus longifilis* were feed on the nutritional requirements of a fish diet formulated at the Fish biology laboratory at the University of Debrecen, Hungary. The formulation was made with pellets of 2.0 mm diameter and contained 64% crude protein and 12% of crude fat.

Preparation of enzyme extract

The fish were sacrificed using ice-cold water with clove oil. The stomach and proximal intestine (including pyloric caeca) were dissected and stored at -20 °C. Crude extracts were prepared through manual homogenization in distilled water (1:10 w/v), followed by centrifugation at 12,000 rpm, 3 °C for 15 minutes. The resulting supernatants stored at -20 °C and the samples were lyophilized until further analysis. The Total protease production in the stomach and intestine was assessed using average values from four fish. The mean protein intake per meal was calculated based on daily feed intake and the approximate composition of the feed.

***In vitro* digestion assay**

A preliminary experiment was conducted to determine the time required for the feed to pass through the stomach and intestine of the fish at three temperatures: 23 °C, 25 °C, and 27 °C. After feeding, the fish were euthanized at different postprandial intervals (0, 0.5, 1, 2, 4, 6, and 8 hours). Each fish was dissected, and the gastrointestinal tract was examined to locate the feed and identify whether it was in the gastric or intestinal phase. The results indicated that the gastric phase lasted approximately 4 hours, while the intestinal phase lasted about 6 hours. The pH was also measured during each phase. In the gastric phase, the pH values were 4.06 at 23 °C, 4.22 at 25 °C, and 3.89 at 27 °C. In the intestinal phase, the pH values were 6.00 at 23 °C, 6.13 at 25 °C, and 6.18 at 27 °C. These findings were later used to establish an *in vitro* digestion model simulating the digestive conditions of the hybrid catfish (*Clarias gariepinus* × *Heterobranchus longifilis*). The system consisted of a two-phase (gastric and intestinal) batch-type digestion unit constructed using small acrylic jacketed chambers adapted for digestion assays (*Figure 1*). The chambers contained stomach and intestinal extracts. The gastric phase was maintained at pH 4.0 for 4 hours, followed by an intestinal phase at pH 6.0 for 6 hours.

Figure 1. ***In vitro* digestion chambers used for assessing protease activity in fish digestion studies**

The chambers contain tissue samples subjected to enzymatic digestion under controlled temperature conditions using a heated platform (Thermo). Yellow screw caps and attached tubing allow sampling of digested material, while the top ports permit reagent addition. This setup simulates gastrointestinal conditions to evaluate enzyme efficiency. Photo: Péter Bársony

Determination of acid protease activity

It was determined according to ANSON (1938), Acid protease activity was determined using haemoglobin (0.5% p/v in buffer) as a substrate. Enzyme activity was measured using a spectrophotometer at a wavelength of 280 nm. It was used Haemoglobin from bovine blood (H-2625, SIGMA). Hb preparation (0.5% p/v): Dissolve 0.75 g

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of glycine and 0.50 g of Hb in 100 mL of water. Adjust the pH to 2 with 1N HCl, and trichloroacetic acid (TCA) in a 20% solution. The assay was conducted in 2 mL microtest tubes, with three repetitions per sample and two repetitions for controls. The substrate was mixed with a buffer (1 mL) with the enzymatic extract (10–20 µL) in three sample tubes. For controls, it was added a substrate with a buffer only in two microtest tubes. The samples were at 25 °C during 20–30 min. The microtest tubes were storage in the fridge for 15 min and centrifugated at 13.000 x g for 15 min. The lectures of the supernatant at 280 nm (UV, special containers). In the case the lectures were made in microplate (corning product #3635) of 96 cups, charge with 200 µl per cup. It was checked the Cytation3 lecture with the program Gen 5. Protease activity was determined according to the following formula:

$$\frac{U}{ml} = \frac{\Delta abs * Vol\,final\,reaction\,(ml)}{CEM * time\,of\,\,incubation\,(\mathrm{min}) * \mathrm{Vextract}\,\,(ml)} * \,dilution\,factor$$

where: ΔAbs represents the increase in absorbance, and MEC represents the molar extinction coefficient of tyrosine (0.008 mL/µg/cm).

Statistical analysis

A one-way ANOVA followed by Tukey's post hoc test ($p < 0.05$) was applied to evaluate enzyme activity results and the simple linear correlation was used to correlate the weight of the stomach and intestines and the enzyme production. Mean nutrient composition values were compared through a univariate analysis of variance with Tukey's test ($p < 0.05$).

RESULTS AND DISCUSSION

RESULTS

The effect of temperature on protease activity in the stomach and intestines of hybrid African catfish (*Clarias gariepinus* × *Heterobranchus longifilis*) was evaluated at 23 °C, 25 °C, and 27 °C. Stomach samples were incubated for 20 minutes to assess enzymatic activity, while intestinal samples were incubated for 15 minutes. Subsequently, protease efficiency (activity per unit of total enzyme) per g, protease activity per millilitre, and the correlation between organ size and enzyme production were determined. Enzyme production in the stomach was highly correlated with organ mass ($R^2 = 0.9095$). A similar trend was found for the intestine, with a high correlation ($R^2 = 0.7028$), although with greater individual variability.

Determination of acid protease activity

In the stomach, protease activity was at 25 °C (3.41 ± 0.16 U/mL), representing a 14.4% increase compared to 23 °C (2.98 ± 0.40 U/mL). However, activity declined by 9.1% when the temperature was raised to 27 °C (3.10 ± 0.31 U/mL). (*Table 1*). The total variation in gastric protease activity across temperatures was 14.4%. Intestinal protease activity was at 27 °C (2.03 ± 0.27 U/mL), the activity was 62.4% higher than at 25 °C (1.25 ± 0.35 U/mL) and 17.3% higher than at 23 °C (1.73 ± 0.51 U/mL) (*Table 2*).

Table 1. **Crude protease activity (Δabs at 280 nm)**

Parameter	Temperature °C			
	23	25	27	P-value
Crude protease (ΔABS 280nm)	0.75 ± 0.10	0.86 ± 0.04	0.78 ± 0.08	0.071
U/ml	2.98 ± 0.40	3.41 ± 0.16	3.10 ± 0.31	0.263
U total	10.36 ± 3.19	4.90 ± 3.13	5.84 ± 2.56	0.107

Enzyme activity (U/ml) and total activity (U) in hybrid African catfish (*Clarias gariepinus* × *Heterobranchus longifilis*) stomach extracts at different temperatures (23 ° C, 25 ° C and 27 °C).

Table 2. **Crude protease activity (Δabs at 280 nm)**

Parameter	Temperature °C			
	23	25	27	P-value
Crude protease (ΔABS 280nm)	0.44 ± 0.13	0.32 ± 0.09	0.52 ± 0.0	0.098
U/ml	1.73 ± 0.51	1.25 ± 0.35	2.03 ± 0.27	0.263
U total	19.55 ± 8.54	8.42 ± 2.25	17.59 ± 7.64	0.153

Enzyme activity (U/ml), and total activity (U) in hybrid African catfish (*Clarias gariepinus* × *Heterobranchus longifilis*) intestinal extracts at different temperatures (23 °C. 25 °C. and 27 °C) (±SD).

Enzyme activity as a percentage of the total (stomach vs intestines)

The distribution of protease activity between the stomach and intestine of hybrid African catfish across the three temperature treatments (23 °C, 25 °C, and 27 °C) had a slight increase at 25 ºC and 27 ºC. These findings may suggest a modest temperature-related enhancement, while the intestine consistently remained the dominant site of proteolytic activity in all temperatures evaluated (*Figure 2*).

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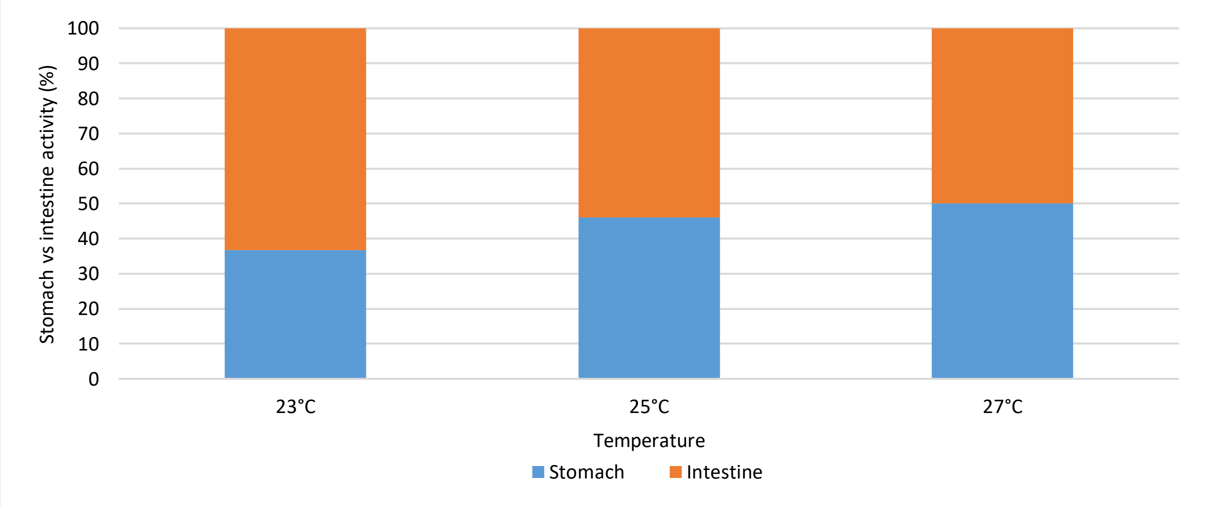
Figure 2. **Stomach vs intestine protease activity (%) in hybrid African catfish (*Clarias gariepinus* × *Heterobranchus longifilis*) across different treatments**



Discussion

This study evaluated the influence of temperature on protease activity in the stomach and intestine of hybrid African catfish (*Clarias gariepinus* × *Heterobranchus longifilis*). Providing important insights into digestive physiology and its implications for nutrition management in aquaculture. No statistically significant differences were observed in protease activity. When the individual stomach and intestine activity was determined it was observed that at different temperatures the protease activity was higher at 27 ºC. The compensation by the intestine is still active in individuals with lower gastric activity, ensuring digestive continuity. As temperature increases, the hybrid African catfish shifts its proteolytic burden upstream (toward the stomach). A higher stomach activity may lead to a faster initial digestion leads to a more efficient absorption downstream. Moreover, the compensatory flexibility remains intact, ensuring performance under physiological variability among individuals.

Since hybrid African catfish are commonly cultured in warm environments, lower water temperatures may reduce digestive efficiency, slow feed intake, and ultimately impair growth performance and feed conversion ratio (FCR). Maintaining optimal rearing temperatures, therefore, is crucial for maximising digestive enzyme activity, feed utilisation, and overall production efficiency. The fish gained the best weight during *in vivo* studies in African catfish larvae, SGR, and survival rate at 28 °C (Orina et al., 2016). In fingerlings, it was registered that the optimal rearing temperature was 26 °C (Ogunji and Awoke et al., 2017). During the egg stage, the optimal temperature was 30 °C (Haylor and Mollah, 1995). During the juvenile stage, a temperature range between 26 and 32 °C did not affect productive parameters (Kasihmuddin et al., 2021). However, in a different study conducted by Al-deghayem et al. (2017), the optimal temperature was 28 °C in hybrid African catfish (*Clarias gariepinus* × *Heterobranchus longifilis*). Investigations related to the effect of different temperatures on feed digestion were unavailable. However, most of the experiments focus on researching the digestibility of different feedstuffs at experimental temperatures ranging between Oellermann (1995) reported a preference for temperatures from 28 °C to 34 °C.

These results suggest that at temperatures above 23 °C and 25 °C, it may be beneficial to formulate diets using highly digestible protein sources. Additionally, supplementing exogenous digestive enzymes such as carboxypeptidase A and B, chymotrypsin, elastase, and chitinase could enhance protein utilisation. Consistent water temperature monitoring is essential to optimise digestive efficiency and nutrient absorption and reduce feed waste.

This study shows that the temperature does not influence the protease activity in stomach and intestine. However, when the percentage of the total gastric digestion was measured it can be observed that at higher temperatures the protease activity is higher in the stomach. In African catfish, digestive and developmental processes are highly influenced by temperature. During the larvae stage, lower temperatures are desirable. Higher temperatures increase fast development and digestion, but it does not necessarily translate to better nutrient absorption. During the hatching phase, 25–26 °C temperatures are commonly used. Lower temperatures tend to slow biological processes, leading to delayed hatching. At these lower temperatures, the fish embryo remains in the egg longer, relying solely on yolk sac reserves for nourishment, being hatched with a small hard yolk sac. If the fish hatches with a relatively large yolk sac, it may be more physically vulnerable to external stressors. Improper handling during this stage can reduce survival rates. Ideally, the yolk sac should be substantial enough to sustain early development but not so large that it hinders mobility or increases

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vulnerability. In African catfish, the yolk sac is typically absorbed within two days post-hatching, at which point exogenous feeding must begin. Extending the yolk sac absorption time by maintaining lower water temperatures may allow for more gradual nutrient uptake, improving digestive efficiency. This strategy can enhance the effectiveness of early feeding. After the initial days, the water temperature can be gradually increased, starting from 25 °C and rising to 27–28 °C by the fourth day of feeding with formulated diets. A more resilient yolk sac and controlled temperature management can support better nutrient absorption and increase the likelihood of survival and growth.

CONCLUSIONS

This experiment demonstrates that temperature changes in hybrid African catfish (*Clarias gariepinus* × *Heterobranchus longifilis*) does not affect protease activity. However, the management of temperature changes along the productive cycle is key in feed formulation and farm management practices, particularly in variable thermal environments. Hence, ensuring optimal temperature conditions can boost fish digestion is important. The highest enzyme activity as a percentage of the total (stomach vs intestines) for protease activity in the stomach and intestine was 27 ºC. However, this does not necessarily mean it is the optimal temperature for overall fish digestion, as the digestive process is highly complex and depends on the synergistic interaction of multiple factors—such as environmental conditions, management practices, and food quality—all of which must be optimized to ensure proper digestion and nutrient digestibility.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

All authors wrote the manuscript, revised, and approved the final version of the manuscript.

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