



One-year old European perch (*Perca fluviatilis*)-specific *in vitro* digestion studies in special regard to feed optimization

Krisztina Takács^{a,*}, Otilia Antal^a, András Nagy^a, Attila Kertész^b, Milán Fehér^c, Péter Bársony^d

^a Department of Nutrition Science, Institute of Food Science and Technology, Hungarian University of Agriculture and Life Sciences, H-1118 Budapest, Somlói road 14-16, Hungary

^b Doctoral School of Animal Science, University of Debrecen, H-4032 Debrecen, Böszörményi str 138, Hungary

^c Department of Animal Husbandry, Institute of Animal Science, Biotechnology and Nature Conservation, Faculty of Agricultural and Food Sciences and Environmental Management, University of Debrecen, H-4032 Debrecen, Böszörményi str 138, Hungary

^d Department of Animal Nutrition Physiology, Institute of Animal Science, Biotechnology and Nature Conservation, Faculty of Agricultural and Food Sciences and Environmental Management, University of Debrecen, H-4032 Debrecen, Böszörményi str 138, Hungary

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ABSTRACT

The aim was to investigate the effect of water temperature on digestive enzyme activities of perch, to determine at which temperature their digestion process is more suitable. European perch (*Perca fluviatilis*) (1 year old, 50–60 g, 10 cm) were kept in recirculation system with the stocking density of 9 kg/m³ in 150 litre-sized-tanks, at 16°C, 18°C, 20°C, 22°C. Fish were kept for 1 week acclimatization, starved for 72 hours before feeding (crude protein: 45 %, crude fat: 16 %, crude fiber: 2.6 %; digestible energy: 18.5 MJ). Feeding was twice, with a 6 hours interval. There were 3 tanks for each proper temperature treatment; every tank contained 100 fish. 10 fish were moved out from all the tanks (30 fish per treatments). Stomach, intestine of fish was separated and their walls were washed with 1 ml of distilled water (pH 2.5), with 1 ml of 50 mM Tris-HCl, containing 0.5 M NaCl, 20 mM CaCl₂ (pH 7.8), respectively, to retrieve gastric and intestinal digestive extracts. Extracts were used for pepsin, trypsin, total alkaline proteolytic, lipase, α-amylase activity tests. We have succeeded in developing protocol for gaining enzyme-containing extracts to monitor the realistic physiological conditions of perch based on enzyme activity measurements. The protocol was considered to be easy to implement and to provide sufficient enzyme extract to simulate subsequent *in vitro* digestion. The ambient temperature affected enzyme activities significantly: pepsin (40480.14; 69245.91; 79305.86; 40236.32 U/mg protein), trypsin – (7.77; 9.88; 11.12; 5.42 U/mg protein), total alkaline proteolytic – (1.47; 1.14; 1.25; 0.84 U/mg protein), lipase – (0.40; 1.16; 0.72; 0.76 U/mg protein) and α-amylase (0.29; 0.395; 0.98; 1.17 U/mg protein) activities, respectively at 16, 18, 20 and 22°C. Based on protease activities, 18–20°C was chosen to be the most appropriate rearing temperature, which contributes the most to the proper growth of the fish.

1. Introduction

Fresh water fish Eurasian perch (*Perca fluviatilis*) is gaining increasing attention in recirculation aquaculture system in Europe. This species is relatively new in aquaculture production, but it is an expanding area of commercial fish farming in Europe. Eurasian perch (*Perca fluviatilis*) and its close relative (only the position of the pre-dorsal bone differs) yellow perch (*Perca flavescens*) (Thorpe, 1977a, 1977b) have a good organoleptic quality, thus they are acknowledged as

valuable food by humans (Geay and Kestemont, 2015). Still, *P. flavescens* and *P. fluviatilis* have no standardized feed yet, although this would be vital for breeding perch for trade (Geay and Kestemont, 2015; Kumar et al., 2019). Due to its carnivorous feeding habits, it is usually reared on high protein diets formulated mostly with marine - made from wild-caught fish - fishmeal for balanced nutritional composition (Stejskal et al., 2020), the yield of which is decreasing (Toviho and Bársony, 2020). This not only limits the accessibility to fish meal, but also increases its price (Henry et al., 2015). Fishmeal prices can represent even

* Correspondence to: Department of Nutrition Science, Institute of Food Science and Technology, Hungarian University of Agriculture and Life Sciences, Department of Nutrition Science, H-1118 Budapest, Somlói road 14-16, Hungary.

E-mail addresses: Takacs.Krisztina@uni-mate.hu (K. Takács), antalotilia@gmail.com (O. Antal), Nagy.Andras.dr@uni-mate.hu (A. Nagy), kertesz@agr.unideb.hu (A. Kertész), feherm@agr.unideb.hu (M. Fehér), barsonp@agr.unideb.hu (P. Bársony).

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40–70 % of fish production costs (Henry et al., 2015). Thus, the currently used fishmeal has to be replaced or supplemented with alternative sources (plants and/or insects). Several alternative feeds have been already tested for yellow perch and European perch, for example wheat gluten (Kamaszewski et al., 2020; Ostaszewska et al., 2013); soybean meals (Kasper et al., 2007); processed soybean meal (Kumar et al., 2019); partially defatted *Hermetia illucens* larva meal (Stejskal et al., 2020); intact baker's yeast (*Saccharomyces cerevisiae*), extracted baker's yeast (*S. cerevisiae*), zygomycetes (*Rhizopus oryzae*) and blue mussel (*Mytilus edulis*) (Langeland et al., 2016); mixture of house cricket (*Acheta domesticus*) and superworm (*Zophobas morio*) (Tilami et al., 2020); defatted microalgae meal (*Haematococcus pluvialis*) combined with soy protein isolate (Jiang et al., 2019); common carp muscle (Schaeffer et al., 2012).

Fishmeal can be replaced or supplemented with plant or insect protein, but several factors should be taken into consideration (e.g., the presence of antinutritive compounds, the ecological footprint, the ratio of different protein sources, the cost and efficiency of processing conditions) during the design of novel fish feed (Henry et al., 2015). Thus, the quality and quantity of a protein source should be optimized to achieve proper biological accessibility (i.e., it becomes potentially absorbable in the intestine) (Brodkorb et al., 2019). The bioavailability of feed components can be increased if the fish are kept under appropriate rearing conditions where digestion is optimal. We can say that the determination of the most advantageous rearing techniques and conditions is a great challenge for the researchers and fish breeders. The process of digestion can be affected by several environmental (water temperature, pH, light-dark cycles), physiological parameters, the diet (feed matrix effect, food quality, quantity)/feeding habits-behaviour, feed intake frequency and also the fish species, size/age; and thus, can influence the biological accessibility/ availability of the feed (Hani et al., 2018; Miegel et al., 2010; Solovyev et al., 2016; Wang et al., 2021). The afore-mentioned factors influence the synthesis, secretion and catalytic properties (activities) of digestive enzymes remarkably (Hani et al., 2018; Miegel et al., 2010), which also change the digestion process (Volkoff and Rønnestad, 2020) and so the fish growth, including the integration of feed protein in fish tissue (through hydrolysis to amino acids in the digestive tract, absorption, transport, and protein synthesis) (Fang et al., 2010; Lemieux et al., 1999; Rungruangsak-Torrisen et al., 2006). The feeding habits of *P. fluviatilis* change on several occasions during its ontogeny: first it is planktivorous, then benthophagous, and when it reaches 14–18 cm it becomes strictly ichthyophagous (Palińska-Żarska et al., 2020). However, 15 mm long *P. fluviatilis* young, small fish can already consume fishes (Solovyev et al., 2014). Since the digestive physiology of the given fish species can adapt to the ambient conditions, the protein bioaccessibility can be optimized by properly adjusted rearing conditions (Nolasco-Soria, 2021). Palińska-Żarska et al. (2020) have evaluated the digestive enzyme activity of wild and domesticated Eurasian perch larvae. The amylase, trypsin and lipase activity were higher in wild larvae, while the pepsin and leucine aminopeptidase activity were higher in domesticated larvae. According

to Palińska-Żarska et al. (2020) this phenomenon can be explained by the adaptation of domesticated larvae to high protein feeds, as wild-living larvae have access to a wide range of zooplankton organisms (with a very different composition).

As one of the main factors that determine the feed protein bio-accessibility is the perch digestive enzyme activity, its knowledge can be useful in designing feeding and rearing protocols. We collected information on the attempts made so far by other researchers on the measurement of digestive enzymes activities of Eurasian perch (*Perca fluviatilis*) and yellow perch (*Perca flavescens*) in Table 1. Research groups used different enzyme activity measurement protocols, which led to different results. This makes the comparison of the results impossible (Brodkorb et al., 2019; Minekus et al., 2014). According to our best knowledge, so far there are no standardized activity measurements of fish enzymes (Moyano et al., 2015), but it would help in interpreting and comparing the results. There is a need to solve this in the future. For this, first of all, in order to properly estimate the effect of ambient temperature on digestive enzymes, we need to take into consideration the anatomy, and the peculiarities of digestive physiology of the investigated fish species. The digestive system of Eurasian perch consists of the mouth, esophagus, pyloric caeca, stomach, liver, gall bladder, pancreas, intestine and anus. In case of *Perca fluviatilis* the stomach is T-shaped, with a wide horizontal part, and a vertical short section, which forms the pylorus at the bottom of the T-shape. The pylorus is followed by the loop-forming intestine. The pyloric caeca can be found (in case of *P. fluviatilis* is made of 3 finger-like projections (Langeland et al., 2013)) at the anterior end of the intestine (De Iuliis and Pulerà, 2019). In teleost adult fish specimens, the enzyme producing pancreas is not a distinct organ, it is represented by a diffuse tissue dispersed “within the mesentery of the anterior stomach section, among the pyloric caeca, and bile duct” (Hamza et al., 2015; Ozkizilcik et al., 1996). The pyloric caeca are blind, finger-like appendages, which originate from the proximal intestine, contain pancreatic tissue, and excrete digestive enzymes, facilitating the digestion of some species (Stevens, 2011). In contrast with mammals, where the parietal cells excrete the hydrochloric acid, while the chief cells secrete pepsinogen, in fish mainly one cell type is responsible for the excretion of both hydrochloric acid and pepsinogen (pepsin). These follicles are found in the stomach (Olsson, 2011). As soon as the digesta enters the intestine, it is mixed with the bile acids that originate from the gall bladder, and this enhances lipase efficiency by emulsification of lipids. Preliminary *in vitro* digestion experiments, which attempt to mimic the physiological conditions in the gastrointestinal tract of fish, may reduce the number of fish that need to be euthanized for biological availability evaluations. Furthermore, *in vitro* digestion models are presumed as more reproducible, faster, and cost-effective tools than *in vivo* assessments.

Our goal was to reveal the effect of the ambient temperature (16–22 °C) on the digestion of Eurasian perch (*Perca fluviatilis*) - that was fed with commercial feed – by monitoring the possible changes in the digestive enzyme activity (pepsin, total alkaline protease, trypsin, lipase, α -amylase) in the gastric and intestinal compartments of perch.

Table 1
Digestive enzyme activities measured in *P. fluviatilis* (1.) and *P. flavescens* (2.).

sp.	Initial weight/length	Length of feeding with different experimental diets	Type of enzyme extract (tissue/digesta)	Type of enzyme	Method of activity determination	Measured activity	Reference
1.	50–70 g	wild-caught	chyme mucosa	peptidases	modified Anson, (1938); pH 7.4, substrate=casein	0.106±0.001 µmol/ (g wet tissue' min.) 0.066±0.002 µmol/ (g wet tissue' min.)	Kuz'mina et al. (2017)
2	32±1 g/ 13.5±0.6 cm	60 days	from mid-part of the intestine	trypsin lipase amylase	BioVision, United States Kit Sigma-Aldrich Kit Sigma-Aldrich Kit	2–4 mU/ml 10–20 mU/ml 20–30 mU/ml	Abd El-Gawad et al., (2019)
1	12- and 18-months old		tissues of mid- & distal intestine	lipase	Albro et al., 1985	~2–12 U/mg protein	Langeland et al., (2013)

The results can be useful in the determination of the rearing temperature that favors digestive enzyme (protease) activity, which can enhance protein bioaccessibility. Still, the costs of maintaining the chosen temperature should also be considered beside the benefits that may be noticed in growth, and fish meat quality due to a higher protein bioaccessibility. The investigation of perch digestive physiology at different ambient temperatures will also support the development of non-invasive *in vitro* protocols (Wang et al., 2021), that can be applied during the research of optimal breeding conditions (feed development, feeding regime).

2. Materials and methods

2.1. Perch fish

Perca fluviatilis (perch) fish were bred by ourselves and reared in a recirculation system till the starting of the experiment. When we set up the experiment the fish were 1 year old, their body weights were 50–60 g, the length were 10 cm. The stocking density was 9 kg/m³. The fish were kept in 150 litre-sized tanks at the Fish Biology Laboratory, University of Debrecen, in different water temperatures (16°C, 18°C, 20°C, 22°C). There were 3 biological replicates for each temperature. In total there were 12 fish tanks and every tank contained 100 pieces of fish. At the end of the experiment 25–25 specimens were collected in each case.

The fish were kept in the system for 1 week for acclimatization. They were starved for 72 hours to empty the digestive tract before feeding. The fish were fed with commercial feed, twice, with 6 hours interval. Feed parameters are the followings: size of the pellets: 3 mm; biochemical composition: crude protein: 45 %, crude fat: 16 %, crude fiber: 2.6 %; digestible energy: 18.5 MJ; ingredients: fish meal, meat meal, soy concentrate, sunflower concentrate, blood meal, wheat, rapeseed meal, fish oil, rapeseed oil. According to the recommendation of the feed producer, the feed quantity was 0.75 % of their body mass, at both times. Sampling fish were anesthetized with 150 mg/l ethyl 3-aminobenzoate methanesulfonate (Cat.No. MS-222, Sigma-Aldrich) after one hour has passed from the second feeding. The above-mentioned *in vivo*-digestion protocol ensured the presence of feed in the stomach and the intestine at the same time, so accordingly a measurable quantity of digestive enzymes was secreted in both digestive phases. This not only facilitates the preparation of extracts with high digestive enzyme activity, it also enables the measurement of the physiological enzyme activity of the digesta in both gastric and intestinal phases at the same time. Then the fish specimens were dissected to retrieve their continuous digestive system. Digestive systems were stored at –20°C until further processing.

2.2. Preparation of perch gastric and intestinal enzyme extracts

The separated, freezed perch fish digestive systems were thawed on ice batteries, at approximately 4°C, so that they could be easily processed. Stomach was separated from the intestine at the line of the pylorus (Kolkovski et al., 2000), then was cut longitudinally as described by Almeida et al., (2018). The stomach was washed with 1 ml of distilled water (pH 2.5; adjusted with 0.1 M HCl) (Kolkovski et al., 2000, Oliveira et al., 2014), to retrieve the gastric crude extracts. The extracts of the 25 specimens kept at different water temperature (16°C, 18°C, 20°C, 22°C) were pooled, respectively. The contents of the intestine were squeezed out manually, and then the intestines were cut longitudinally. The intestine wall was washed with 1 ml of the physiological buffer recommended by Fuchise et al., (2011), namely 50 mM Tris-HCl, which contains 0.5 M NaCl and 20 mM CaCl₂ (pH 7.8). In case of each investigated water temperature (16°C, 18°C, 20°C, 22°C) the extracts of 30 specimens were pooled. The pooling of gastric and intestinal extracts, respectively, that was sampled from 30 individual fish was an important step in order to obtain homogenized sample and sufficient quantities for

all enzyme activity tests. The crude extracts were homogenized with Ultra-Turrax T25 (30 sec) in beakers (Oliveira et al., 2014; Villanueva-Gutiérrez et al., 2020), then by mixing with magnetic stirrer (BioSan MS 3000) for 1 hour in refrigerator (4°C) in order to facilitate the extraction of the enzymes. The homogenized crude extracts were then centrifuged (4100 rpm, 10 minutes, 25 °C). The aliquots of supernatants were then transferred into 2 ml-eppendorf tubes, then further centrifuged (12,000 rpm, 15 minutes, 4°C) in order to discard the cell debris, lipids, feed, and other materials from the sample. The supernatants containing digestive enzymes, later called as perch digestive fluid, were stored at –20°C until further analytical measurements.

2.3. Digestive enzyme activity measurements

Pepsin activity measurement of gastric enzyme extract was made according to Anson (1938). Intestinal enzyme extract was used to measure trypsin - (Hummel, 1959), lipase - (Carriere et al., 1993) and α -amylase - (Bernfeld, 1955). All these enzyme activity protocols are described in detail in a publication by Brodkorb et al. (2019), which is here described as followings:

i, pepsin activity measurement: In the presence of pepsin, TCA-soluble tyrosine peptides are generated from haemoglobin and H₂O. One unit of pepsin produces a change in absorbance of 0.001 per min at pH 2.0 and 37°C, measured as TCA-soluble products at 280 nm. The substrate is 2 % (wt/vol) haemoglobin in water at pH 2. In this study the enzyme solution was the gastric extract of perch, which was diluted 20 times in buffer made of 10 mM Tris, 150 mM NaCl (pH=6.5). The resulting solution was further diluted in 10 mM HCl in the range of 20-times – 2.9 times. 500 μ L of haemoglobin is mixed with 100 μ L of each enzyme solution and incubated for 10 min at 37°C. To stop the reaction, 1 ml of 5 % (wt/vol) TCA is added. The solution is centrifuged at 6000 g for 30 min at room temperature and the absorbance is read at 280 nm in quartz cuvettes.

ii, trypsin activity measurement: In the presence of trypsin, p-toluene-sulfonyl-L-arginine methyl ester (TAME) and H₂O produces p-toluene-sulfonyl-L-arginine plus methanol. One unit hydrolyzes 1 μ mol of p-toluene-sulfonyl-L-arginine methyl ester (TAME) per min at pH 8.1 and 25°C. Thus, the substrate is 10 mM TAME in H₂O. The enzyme solution in this case was the intestinal extract of perch. 1.3 ml of 46 mM Tris-HCl and 11.5 mM CaCl₂ buffer (pH 8.1) was mixed with 150 μ L of the substrate at 25°C. 50 μ L of diluted intestinal extract (dilution in 1 mM HCl) was added. The absorbance increase was read at 247 nm during 10 min.

iii, lipase activity measurement: Tributyrin plus H₂O produces butyric acid and sn-2 monobutyrin when lipase is applied. One unit releases 1 μ mol of butyric acid per min at 37°C at the pH of the assay. The substrate is tributyrin. The enzyme solution in this experiment is the intestinal extract of perch. The assay solution is 36 mg/l Tris-(hydroxymethyl)-aminomethane, 2 g/l sodium taurodeoxycholate, 9 g/l NaCl and 200 mg/l CaCl₂(H₂O)₂. At 37°C, 14.5 ml of assay solution was mixed with 0.5 ml of tributyrin, and stirred until it formed a fine oil-in water emulsion. 10–100 μ L of intestinal extract was added and the rate of titrant (0.1 N NaOH) to maintain pH 8 for 10 minutes was monitored.

iiii, amylase activity measurement: Starch plus H₂O produces reducing groups (e.g., maltose) when α -amylase is applied. One unit releases 1.0 mg of maltose equivalent from starch in 3 min at pH 6.9 and 20°C. The substrate is 1.0 % (wt/vol) soluble potato starch in 20 mM sodium phosphate buffer with 6.7 mM NaCl, adjusted to pH 6.9. Enzyme solution was the intestinal extract obtained from perch. 0.1 ml of substrate was incubated at 20°C, then the diluted intestinal extract (50–100 μ L) was added and the mixture was incubated at 20°C for 3 min. The reaction was stopped with 0.1 ml of colour reagent (96 mM 3,5-dinitrosalicylic acid, 1.06 M sodium potassium tartrate). The volume is brought to 1.2 ml with H₂O, the tube is capped and boiled for 15 min. The absorbance was read at 540 nm. The activity was calculated against a maltose standard curve.

The total alkaline protease activity of intestinal enzyme extract was measured in compliance with multiple protocols (García-Ortega et al., 2000; Peña et al., 2015; Walter, 1984). Regarding the total alkaline proteolytic activity measurement: 1 Unit enzyme activity means that 1 mg tyrosine is released from the hydrolysis of casein in one min. (280 nm; pH 8.5; 37°C). For the preparation of the tyrosine calibration curve, and for casein hydrolysis measurement we used the following

buffer: 0.1 mol Tris-HCl, 0.01 M CaCl₂ (pH 8.5). Briefly, 325 µl 1 % casein, was mixed with 325 µl buffer, and 50 µl enzyme extract; then incubated at 37°C for 20 mins. The reaction was stopped by the addition of 700 µl 8 % trichloroacetic acid (TCA). The mixture was chilled on ice for 15 minutes, then centrifuged at (12000 rpm, 10 minutes, 4°C). The absorbance of the supernatant was measure in a quartz cuvette at 280 nm (light path= 1 cm).

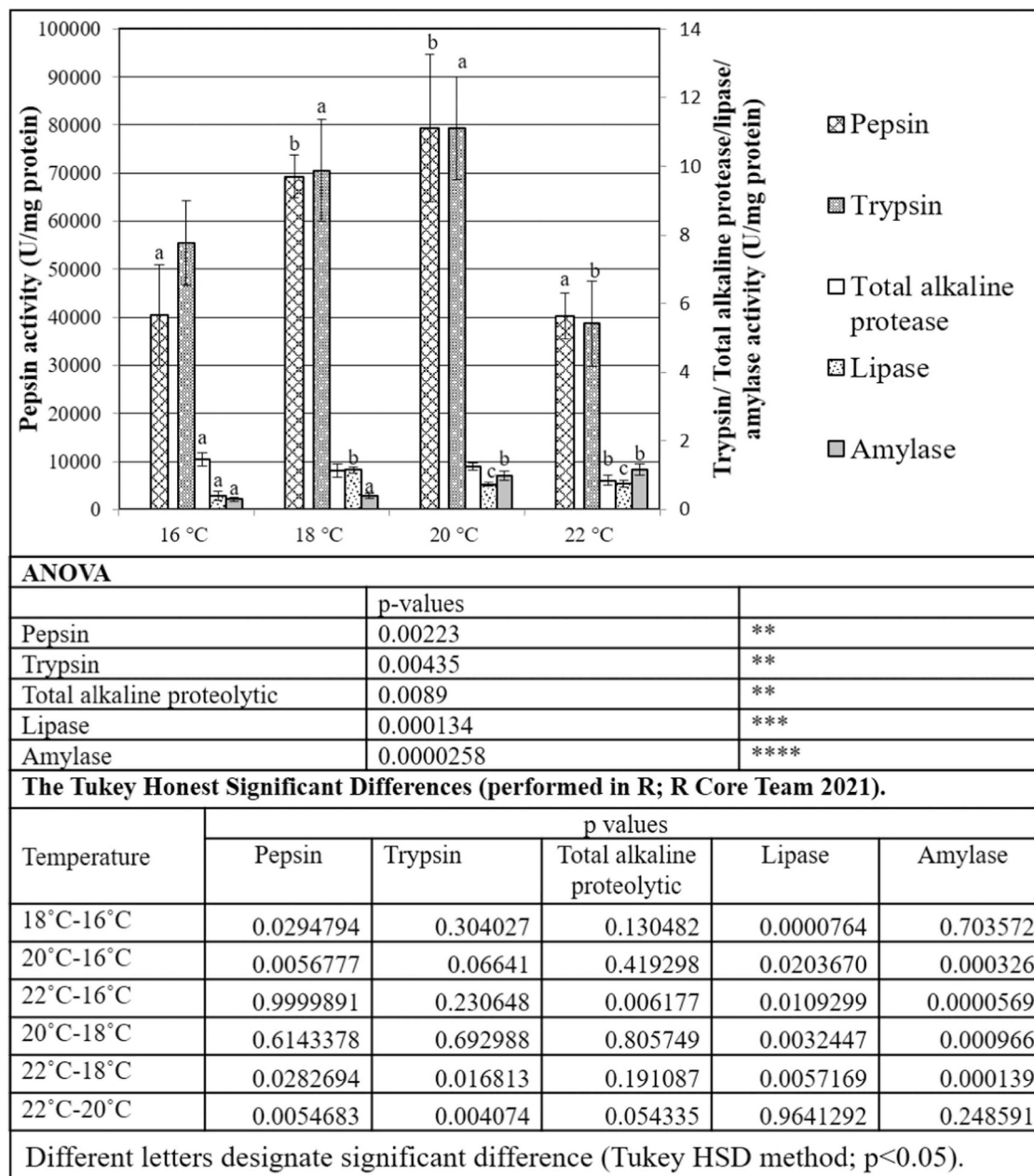


Fig. 1. Digestive enzyme activity measurements of perch (*Perca fluviatilis*) kept at different ambient temperatures. Activities were determined according to the followings: The TCA-soluble products of one unit of **pepsin** will produce a ΔA_{280} of 0.001 per minute at pH 2.0 and 37°C (“Anson” pepsin units). One unit of **trypsin** degrades 1 µM of p-toluene-sulfonyl-L-arginine methyl ester (TAME) in 1 min, at 25°C, pH 8.1. In case of **total alkaline proteolytic** activity measurements one unit liberates 1 mg of tyrosine in 1 min, at 37°C, pH 8.5 (Walter, 1984). At 37°C and pH 8.0 one unit of **lipase** releases 1 µM butyric acid in 1 min. One unit of **α-amylase** at 20°C; pH 6.9 releases from starch in 3 min 1 mg of maltose. These results were normalized with the protein content (Bradford, 1976). **Conclusions on the activities:** Pepsin activity was the highest at 18°C and 20°C, while there was no significant difference in trypsin and total alkaline proteolytic activity at 16°C, 18°C or 20°C. Thus, in case of protease activity we found that 18°C, and 20°C, are optimal for perch breeding. The lipase activity was significantly higher at 18°C, than at 16°C, 20°C or 22°C, while amylase activity was significantly lower at 16°C and 18°C than at 20 and 22°C. **ANOVA tests** were applied to determine whether the environmental temperature has a significant effect on pepsin, trypsin, total alkaline proteolytic, lipase and α-amylase activity, respectively. As all p values were lower than 0.05, we concluded that each investigated digestive enzyme activity is significantly affected by ambient temperature. **Tukey Honest Significant Difference (Tukey HSD method)** determines whether there is significant difference (labelled with different letters on the graph) between the investigated groups of perch, kept at 16°C, 18°C, 20°C or 22°C.

In each case, the enzyme activity of the pooled samples (3 pools of 25 specimens for each ambient temperature (16°C, 18°C, 20°C and 22°C)) in three replicates was measured. The enzyme activities were corrected with the protein content of the enzyme extracts, so results were given in Units/mg protein. Then, the average enzyme activity of the pools was calculated from the technical replicates.

2.4. Determination of protein concentration of digestive enzyme extracts

We applied the Bradford (1976) method to determine the protein content of the gastric and intestinal supernatant samples, utilizing the Bradford reagent (Sigma B6916) according to the given protocol. Results were given in mg protein/ml supernatant concentration.

2.5. Statistical analysis of enzyme activity measurements

Shapiro-Wilk Normality Test and Bartlett Test of Homogeneity of Variances in R (R Core Team, 2021) was used to verify the normality and homogeneity of variances of the results.

ANOVA tests were applied (R Core Team, 2021) according to Alkharbi and Alqaraghuli (2019) to find out whether the environmental temperature has a significant effect on pepsin, trypsin, total alkaline proteolytic, lipase and α -amylase activity, respectively. Tukey Honest Significant Difference method in R (R Core Team, 2021) was used to determine whether there is a significant difference between the effect of 16°C, 18°C, 20°C and 22°C regarding pepsin, trypsin, total alkaline proteolytic, lipase and α -amylase activity.

3. Results

3.1. The effect of ambient temperature on the perch digestive enzyme activities

The knowledge of the activity of digestive enzymes can be useful in designing feeding and rearing protocols. One of the main factors that determine the feed protein bioaccessibility is the perch digestive enzyme activity. The feeding regime and the timing of fish slaughter were chosen in a manner that enables the presence of feed in the stomach and intestine at the same time. During these preliminary experiments, the application of these adjustments and the implementation of a proper extraction procedure (described in subsection 2.2) was noticed to be indispensable for retrieving digestive enzyme extracts effectively (with the proper yield for analyses). All enzyme activity were significantly dependent on the ambient temperature (concerning all of the investigated temperatures) according to the ANOVA test (Fig. 1.).

The pepsin activity was the lowest at 16°C and at 22°C (40480.14 and 40236.32 U/mg protein, respectively). At 18°C it increased to 69245.91 U/mg protein, while at 20°C it reached an average of 79305.86 U/mg protein. The pepsin activity at 18°C and 20°C is significantly higher than the one measured at 16°C and 22°C (TukeyHSD, $p < 0.05$).

Trypsin activity increased progressively (but not significantly) between 16 and 18–20°C (it was 7.77; 9.88; and 11.12 U/mg protein), then it was diminished at 22°C to 5.42 U/mg protein.

The trypsin activity of the Eurasian perch is significantly (TukeyHSD method, $p < 0.05$) higher at 18°C and 20°C, than at 22°C. The trypsin activity at 16°C was lower than in case of perch kept at 18°C or 20°C. In case of perch kept at 16°C or 22°C, the trypsin activity did not differ significantly.

Total alkaline proteolytic activity was 1.47 U/mg protein at 16°C; 1.14 U/mg protein at 18°C; 1.25 U/mg protein at 20°C; and 0.84 U/mg protein at 22°C. When perch were kept at 16°C, the total proteolytic activity was significantly higher than at 22°C, according to the TukeyHSD test ($p < 0.01$). There was no difference in total alkaline proteolytic activity at 16°C, 18°C and 20°C with TukeyHSD test. 18°C, 20°C and 22°C also did not differ significantly according to TukeyHSD

method.

We measured the highest lipase activity, 1.162 U/mg protein at 18°C. The lipase activity of the intestinal extract of fish kept at 16°C was 0.40 U/mg protein. The lipase activity at 20°C and 22°C was very similar, being 0.72 U/mg protein and 0.76 U/mg protein on average. The lipase activity at 18°C was significantly higher (TukeyHSD Test, $p < 0.01$) than at 16°C, 20°C and 22°C. Although the lipase activity at 20°C and 22°C was lower than at 18°C, it was still significantly higher (TukeyHSD method, $p < 0.05$) than at 16°C.

The amylase activity increased between 16 and 22°C, from 0.29 U/mg protein at 16°C to 1.17 U/mg protein at 22°C. The amylase activity measured at 16°C, was not significantly different from the one at 18°C, which was 0.395 U/mg protein. At 20°C, the amylase activity was 0.98 U/mg protein and did not differ significantly from the average activity at 22°C. The perch kept at 20°C and at 22°C had significantly higher α -amylase activity than those that were cultured at 16°C and at 18°C (TukeyHSD Test $p < 0.001$).

3.2. Utilization of data on digestive enzyme activity in the development of an *in vitro* perch-specific digestion model

We intend to develop a static perch fish *in vitro* digestion model to use as a preliminary evaluation of the biological value of perch feed. *In vitro* digestion models attempt to mimic the physiological conditions in the gastrointestinal tract of fishes. Currently, there is no standardized *in vitro* digestion model for any fish species in general or for Eurasian perch. Wang et al., (2021) worked on modelling fish digestion, and their recommended static fish digestion protocol was based on the theory of the human INFOGEST (COST INFOGEST FA1005 Action „Improving health properties of food by sharing our knowledge on the digestive process”, Brodkorb et al., 2019; Minekus et al., 2014) protocol. Application of commercial purified mammalian enzymes could be a possible approach to perform *in vitro* digestion. Although this strategy avoids the sacrifice of fish for *in vitro* experiments, the opinions about its *in vivo* relevance are contradictory (Wang et al., 2021). So, the application of enzyme extracts from the investigated fish species is considered to be a better reproduction of the *in vivo* digestion (Wang et al., 2021). In most of the studies crude digestive enzyme extracts are used to digest the feed. These extracts are that supernatants, which originate from the homogenization and centrifugation of gastric and intestinal digesta (Rahmah et al., 2014; Velazco-Vargas et al., 2014). *In vitro* digestion can be modelled with perch gastric and intestinal fluids, in which the activity of the containing digestive enzymes are known (since they were preliminarily measured as mentioned before). So, after *in vitro* digestion, the results of the analyses on the digestibility/bioaccessibility of any macro or micro components of feed can be well interpretable and informative. According to the current digestive enzyme activity measurements, digestive fluids of specimens kept at 18°C or 20°C are recommended to apply for simulating digestion *in vitro*.

4. Discussion

According to our knowledge, there aren't any *in vitro* digestion protocols for perch described in the scientific literature. Those who have measured digestive enzyme activity in perch, had different goals than us. Kuz'mina et al. (2011) and Kuz'mina and Zolotareva (2019) investigated the effect of pH on the activity of glycosidases and proteinases. Kuz'mina et al. (2012) evaluated the effect of the reaction temperature on proteinases, while Kuz'mina et al. (2017) investigated the effect of phenol and its derivatives on the activity of fish intestinal peptidases. Kumar et al. (2019) monitored the effect of soybean meal on digestive enzyme activity. Kolkovski et al. (2000) and Abd El-Gawad et al. (2019) determined the effect of diet supplementation on digestive enzyme activity. Langeland et al. (2013) compared the activity of digestive enzymes in different regions of the gastrointestinal tract. Golovanova (2010) investigated the effect of Cu and Zn on carbohydrase activity.

The methods for enzyme activity determination in case of the above-mentioned articles were different than the protocols we applied, so the results can't be compared.

In vitro digestion is a complex process, and it is hard to reproduce it. Fish metabolism depends on the ambient temperature, because fish are poikilothermic. Water temperature is among the most important environmental factors, which affect fish (Li et al., 2023). The determination of the optimal temperature for fish has a practical advantage for fish breeders. According to Flogbé and Kestemont (2003), for 18,9 g Eurasian perch the optimum feeding level at 23°C is 2,2 % biomass/ day; while for 1,56 g perch it is 4,5 % biomass/ day. In contrast, for 10 g carp the daily intake level is 9 %. As the daily intake level is lower in perch than in other fish it is of particular importance to choose environmental conditions in a manner that enhances digestive capacity. The digestion can be optimized by keeping perch at the proper temperature.

The perch feeds are very high in protein and fat too. The general values of the feed at the 50 g weight fish are 56 % protein and 18 % fat. Above 50 g of fish weight, the protein reduces to 54 % but the fat increases and reaches 20 %. Because of the very high level of protein the price of a perch feed compared to the trout feeds (which contains less protein (40–45 %)) much higher. If the digestion and the utilization of the nutrient matters will be better we can reduce the protein content of the feed which makes the feed cheaper. It could be a huge advantage for the farmers who can decrease their production costs.

The detection of total alkaline proteolytic activity includes the evaluation of the activity of several proteases (casein-lytic proteases such as trypsin, chymotrypsin, elastases, collagenases, erepsin (mixture of aminopeptidases, di- and tripeptidases), carboxypeptidases, intestinal microbiota proteases (Fänge and Grove, 1979; Holicky, 1989; Kenyon, 1925; Kuz'mina, 2008; Kuz'mina et al., 2015; Shreeve, 2009; Peña et al., 2015). According to Einarsson (1993) the most important endopeptidases in the fish digestive tract are trypsin and chymotrypsin; while the less dominant - because of their low amount, if they are present at all - are the other endopeptidases (e. g. elastase, collagenase). That is why trypsin was selectively and specifically measured as well. According to the summarized results related to proteases, 18°C and 20°C are the most appropriate for maximal protease (pepsin, trypsin, total alkaline proteolytic) activity. According to Tahir et al. (2024) in *Labeo rohita*, when the water temperature increases from 25°C to 35°C the protease activity becomes higher. In contrast, in our case pepsin and trypsin activity is significantly lower at 22 °C, than at 18 °C and 20 °C; and total proteolytic activity is significantly lower at 22°C than at 16°C. Ahmad et al. (2014) and Sharma et al. (2017) noticed similar tendencies as Tahir et al. (2024) in *Clarias batrachus* and *Catla catla*. Similarly to our results, Bowyer et al. (2013) noticed that enzyme activities decreased at higher temperatures in marine fish.

Generally, in some carnivorous species (like perch), only low carbohydrase activities (α -amylase activities) can be detected (Golovanova, 2010; Golovanova et al., 2013; Langeland et al., 2013) as perch usually consumes invertebrates and fishes. It is generally considered that, the lipase activity of perch is more relevant than the carbohydrase, due to the fact that carnivorous fish consume a diet enriched in fat, and they are able to better digest lipids, than carbohydrates (Langeland et al., 2013; Taşbozan and Gökçe, 2017). We detected the highest lipase activity at 18°C, while 20°C and 22°C were more favourable to amylase activity.

While in our case amylase activity increased at higher temperatures, Tahir et al. (2024) noticed the opposite effect in case of *Labeo rohita* at 25°C, 30°C and 35°C. Tahir et al. (2024) also noticed that lipase activity is not affected by temperature. In our case, lipase activity was higher at 18°C, than at 16°C, 20°C and 22°C. The discrepancies in the results of different studies can be explained by the fact that they investigated different fish species.

Although sisorid catfish (*Glyptosternum maculatum*) is distant in taxonomy from perch, its feeding habits are similar (it is also carnivorous). In case of those sisorid catfish, which were caught from the wild at

12°C –14°C, the lipase activity of was 0.61 U/mg protein (Xiong et al., 2011), while lipase activity in our experiment was 0.399 U/mg protein at 16°C; 1.162 U/mg protein at 18°C; 0.72 and 0.76 U/mg protein at 20 and 22°C, respectively. According to our results there is no linear relationship between temperature and lipase activity.

Unfortunately, in the case of fish digestive enzyme activity measurements, different research groups use distinct protocols and the definition of one unit is not standardized, and this impedes the comparison of results. We are facing the same obstacle if we wish to compare the results of Xiaong et al., (2011) with our data regarding lipase activity. We measured a significantly higher lipase activity at 18°C than in case of other investigated ambient temperatures (16°C, 20°C, 22°C). So, 18°C is more advantageous for lipase activity enhancement to improve digestion.

Summarizing all enzyme activity measurements, we proved that 18°C and 20°C gave better (higher) enzyme activity results in case of pepsin, trypsin and lipase. Only amylase activity was significantly lower at 18°C than 20°C, but due to the feeding habits of perch, protease and lipase activity is regarded more important. The higher protease and lipase activity could contribute to a more effective feed digestion process. If we also consider expertise of the technological /industry management practices, cold 18°C would be suggested as the most advantageous rearing temperature (i.e., cold water has better O₂ storing capacity (Harvey et al., 2011)). The process of digestion can be affected by several environmental (water temperature, pH, light-dark cycles), physiological parameters, the diet (feed matrix effect, food quality, quantity)/feeding habits-behaviour, feed intake frequency and also the fish species, size/age; and thus, can influence the biological accessibility/ availability of the feed (Hani et al., 2018; Miegel et al., 2010; Solov'yev et al., 2016; Wang et al., 2021). The determination of *in vitro* protein accessibility can facilitate the estimation of the biological value of feed, or the establishment of more efficient rearing protocols. Our standardized enzyme activity measurements will also be the starting point in the development of a perch-specific *in vitro* digestion model.

5. Conclusion

The costs of feed and rearing conditions have to be chosen in a manner that the expenses: fish meat quantity (and quality) is the most favourable. The objective of our experiments was to gather data about perch physiology that support cost-effective choice of breeding conditions.

Results on activities of perch digestive enzymes support the implementation of rearing conditions (water temperature) that improve protein digestibility. The cost-effective rearing temperature for perch was 18–20°C based on our results. Results regarding digestive enzyme activity of perch also represent the basis of the development of a perch-specific *in vitro* digestion model. This approach can accelerate innovations regarding fish feed development and husbandry. For example, evaluating the effectiveness of alternative protein sources as feed components becomes cheaper, faster and easier if the candidates are screened with the *in vitro* digestion protocol.

Author agreement

According to the common agreement with the co-authors, I am submitting the enclosed material for possible publication in Aquaculture Reports. I declare, that this article has not been published previously and it is not under consideration for publication elsewhere.

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CRediT authorship contribution statement

Krisztina Takács: Writing – original draft, Supervision, Methodology. **András Nagy:** Methodology. **Otilia Tamara Antal:** Writing – original draft, Methodology, Investigation. **Milán Fehér:** Methodology, Conceptualization. **Attila Kertész:** Methodology. **Péter Bársony:** Writing – review & editing, Writing – original draft, Resources, Project administration, Methodology, Investigation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

Data Availability

Data will be made available on request.

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