Role of Peptidases of the Enteric Microbiota and Prey in Temperature Adaptations of the Digestive System in Boreal Carnivorous Fish

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Role of Peptidases of the Enteric Microbiota and Prey in Temperature Adaptations of the Digestive System in Boreal Carnivorous Fish

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Abstract– For the first time the activity of casein-lytic and hemoglobin-lytic peptidases of intestinal mucosa, chyme and enteric microflora of carnivorous boreal fish (pike, zander, burbot, perch) was investigated across a wide temperature range (0–70°C) to reveal the role of the enzymes of the enteric microbiota and the prey in the temperature adaptations of the digestive system of these fish. It was shown that in summer at 0°C, the relative activity of peptidases of intestinal mucosa (less than 20%) is usually considerably less than that of chyme and enteric microflora peptidases (up to 40% of maximal activity). In winter, on the background of low relative activity of mucosa and enteric microbiota peptidases at 0°C revealed a high level of the relative activity of burbot and pike chyme peptidases (45 and 80% of maximal activity). The role of enteric microbiota and prey peptidases in digestive system adaptations of piscivorous fish to low temperatures is discussed.

Keywords: carnivorous fishes, peptidases, intestine, mucosa, chyme, enteric microbiota, potential prey

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INTRODUCTION

Previously, we stressed that many fish enzymatic systems possess limited adaptations to low temperature; however, little data are available to judge whether enzymes of fish prey and enteric microbiota can mitigate this deficiency [20]. At the same time in last decades it was shown that efficiency of trophic relationships of various animals, in particular fish, to a large extent depends on the features of the functioning of enzyme systems of trophic partners and enteric microbiota [19, 35]. It is important to note that the role of prey proteinases (peptidases) in processes of digestion in fishes is still being discussed since the middle of XX century [15]. The results which evidence of participation of prey enzymes in the degradation of proteinaceous components of their tissues have been received in a number of works [8, 9, 15, 18, 24]. However the possibility of the participation of enzymes of preys in processes of digestion in consumers was called into doubt in some works [17, 28].

After the description of the mechanism of the induced autolysis interest to the questions, concerning the contribution of enzymes of a prey to process of digestion in consumers had increased. It is assumed that an important role in structural degradation of fish food items is played by their lysosomal enzymes [18, 35]. The role of lysosomal enzymes (numerous cathepsins) is most significant at the initial stages of digestion in the stomach, while prey integuments have not yet been lysed. During this stage, hydrogen ions penetrate into prey tissues much faster than stomachal proteinases and trigger induced autolysis. It was showed that at pH 2–3 the total peptidase activity in the whole body of fish prey can exceed 5–10 time the total activity of stomach peptidases (mainly pepsin) of a consumer due to the induced autolysis realizing in the tissues of a prey [18].

Microorganisms are entered to the digestive tract at the beginning of larva exogenous feeding and form the indigenous microflora. During the ontogenesis transient microflora was formed in fish digestive tract by ingestion of food or water [5, 19]. Unlike enough stable attached bacterial assemblages, composition of cavitary assemblages is intimately connected with they changes in water and diet [5, 11, 21, 32]. It is known that many strains of enteric and associated microorganisms are produced hydrolases [2, 11, 21, 32], which can participate in symbiotic digestion [22, 34]. However now it is impossible to estimate correctly the contribution of symbiotic microflora enzymes in the digestive processes in fish because of some technical difficulties.

Since it is virtually impossible to directly quantify the contribution of enzymes of the symbiotic microflora to fish digestion, it has been proposed that their role in digestion can be estimated by comparing the

characteristics of enzymes synthesized by microorganisms and the fish digestive system [35]. It is well known that rate of physiological and biochemical processes [13, 14], in particular the activity of digestive enzymes in fish depends on temperature [12, 20, 29, 30, 35]. It is necessary to notice specially that in the conditions of low temperature only the enzymes of consumers providing the initial stages of hydrolysis of biopolymers, in particular, pancreatic by origin α-amylase and peptidases synthesised in fish stomach can function effectively. For the reason that serine proteinases functioning in consumer intestine, do not possess the adaptations, in terms of activity, to low temperature, it was suggested a possible compensatory role of enzymes of the preys and enteral microbiota [35]. At the same time the information, concerning the effect of temperature on enzyme systems of intestinal mucosa, potential food items of fish and enteric microbiota, prior to this work were fragmented [19]. It should be noted that in the above studies the integral indicator of proteolytic activity, and also the natural substrates was specifically used to compare the characteristics of fish enzymes and enteric microbiota, which are functionally similar but differ in structure.

The aim of the work is to study of temperature-dependent characteristics of the peptidases that function in the intestine of some species of carnivorous boreal fish (enzymes of the mucosa, chyme and enteric microbiota) to reveal the role of the enzymes of the enteric microbiota and the prey in the temperature adaptations of the digestive system of these fish.

MATERIALS AND METHODS

Objects of research: carnivorous boreal fish zander *Zander lucioperca* (L.) weight 460 ± 52 g and pike *Esox lucius* L. weight 436 ± 48 g, burbot *Lota lota* (L.) weight 528 ± 67 g and benthivorous-facultative carnivorous perch *Perca fluviatilis* L. weight 448 ± 52 g. The fishes were captured in Rybinsk Reservoir (58°22′30″ N, 38°25′04″ E, Russia) in summer (burbot in winter, pike in summer and winter). Fish were caught by the nets. Alive fish were selected and transported to the laboratory in the containers with water for 1 h. In the laboratory fish were washed with clean water and dried. Then the fish surface treated with alcohol. The abdominal cavity was cut aseptically. To prevent any changes of microflora and any loss of intestinal content the guts were ligated. The intestines were cleaned of exterior fat and cut longitudinally. The intestinal mucosa, the chyme and the colonies of the microflora isolated from the chyme (see below) were used as enzyme active preparations. In summer water temperature in a reservoir was close to 20–22°C. In winter water temperature in a reservoir was close to 0.5°C.

The microflora was isolated according to the Matheis's method [23]. An aliquot (approximately 0.1 g) of the chyme from medial part of intestine placed in a sterile flask on a liquid medium (100 mL of meat-peptone broth). Then, an equal amount of the flask contents of 5 fishes was transferred into another sterile flask and one sample was formed according to the method of mixed samples [33]. Then, 1 mL of chyme diluted 1000 times was taken from the pooled chyme sample and placed on 100 mL of the liquid nutritious medium of beef extract broth. Under such condition the activity of the hydrolases of consumers was practically absent. Flasks were transferred in a thermostat and cultured at 28°C for 48 h at constant mixing.

After sampling microbiota with the help of a special scraper and a small glass spatula (5 mm) a chyme was carefully collected, and it was mixed thoroughly. The aliquots of chyme were selected and weighed. The intestine mucosa was flushed with cooled Ringer solution (3–4°C) for poikilothermic animals (103 mmol/L NaCl, 1.9 mmol/L KCl, 0.45 mmol/L $CaCl_2$, 1.4 mmol/L $MgSO_4$, pH 7.4). Then mucosa was dried with a filter paper and taken out with another plastic scraper, thoroughly collected. The material from 5 specimens (irrespectively of the gender) was combined and mixed thoroughly at the ice bath. The aliquotes of the samples were weighed and homogenized in a glass homogenizer with a small amount of the same Ringer solution at a temperature of 3–4°C. Then the homogenates of mucosa and chyme were diluted with Ringer solution ten times (the end-point dilution was 1 : 99). After homogenates was adjusted to pH 7.4, using a "Besic 20 pH-meter". The samples were examined in summer (15 samples) and in winter (10).

Proteolytic activity was assayed by the increase in tyrosine concentration using the casein or hemoglobin (10 g/L), as substrates, prepared on the same Ringer solution (pH 7.4), at temperature 0–70°C. The casein-lytic activity (CA), and hemoglobin-lytic activity (HA) in the fish and enteric microbiota was determinated. The substrates and homogenates (pH 7.4) were incubated for this purpose during 30 min. The reaction was stopped by adding 1 mL of 0.3 N trichloroacetic acid (TCA). After 10 min, the incubate mixture was filtered by using paper filter. Then it was mixed 0.25 mL of the filtrate, 2 mL of 0.5 N NaOH, 0.25 mL of 0.025 N $CuSO_4$ and 0.75 mL of Folin reagent, diluted in 3 times *ex tempore*. To determine the initial content of tyrosine in samples (background) TCA was added to the homogenate prior to the addition of substrate. The other operations were identical. The concentration of tyrosine in the samples was determined after 30 min. The intensity of colour was measured with the help of photocolorimeter KFK-2 (Russia) at 670 nm. The enzyme activities were determined in five replicates for each point (taking into account of the initial amount of tyrosine in a sample) and expressed as μmol/(g · min).

The results were statistically processed with the use of a standard software package (Microsoft Office' 2007 supplement Excel). The significance of the dif-

ferences between the arithmetical means (M ± m) was estimated using the Student's test for small samples at $p \le 0.001$.

RESULTS

The data, concerning the effect of temperature on CA of fish intestinal mucosa, chyme and enteral microbiota in summer are presented at the Fig. 1. At 20°C intestinal CA range from 0.73 ± 0.08 in the perch to 4.01 ± 0.01 μmol/(g min) in pike, $p \le 0.001$, chyme—from 2.19 ± 0.30 in the perch to 2.62 ± 0.03 μmol/(g min) in zander. As it shown at the figure maximum CA of intestinal mucosa and chyme in all fish species is marked at the temperature 50°C. At 0°C relative enzyme activity of mucosa and chyme usually varies in the range 10–20% of the maximal activity. The highest relative CA is revealed in chyme of perch (38% from a maximum activity). At 20°C values of CA of enteric microbiota in perch is 0.31 ± 0.02, in zander is 0.33 ± 0.07, in pike is 0.80 ± 0.10 μmol/(g min). The differences between enzyme activity in fish of family Percidae and pike are significant, $p \le 0.001$. It ought to be remarked, the methodical distinctions, in particular microbiota cultivation, these values are non-comparable with a CA resulted above. The maximum activity of enteric microbiota CA in all fish species is 50°C. At 0°C minimum of relative enzyme activity of enteric microbiota is in zander (9.4%), the maximum is in the perch (35% of the maximal activity).

In summer at 20°C the minimum of intestinal mucosa HA was found in pike, maximum in zander: 0.26 ± 0.02 and 0.40 ± 0.01 μmol/(g min), $p \le 0.001$, chyme—in zander and pike: 0.40 ± 0.02 and 0.50 ± 0.11 μmol/(g min), respectively (Fig. 2). The temperature optimum HA in all fish species is 50°C. At 0°C the relative HA in all investigated preparation are usually lower than that of CA. The maximum value of the relative HA identified for perch chyme (34% of the maximal activity). At 20°C the values of enteric microbiota HA in zander and perch are 0.33 ± 0.08 and 0.93 ± 0.09 μmol/(g min), respectively, $p \le 0.001$. The temperature optimum in these fish corresponds to 50°C. The highest values of relative enteric microbiota HA at 0°C found in perch (23.7% of the maximum activity).

In winter, at 20°C CA of pike chyme is significantly higher than that in the mucosa: 3.79 ± 0.09, 1.58 ± 0.04 and 1.12 ± 0.15 μmol/(g · min). The differences between enzyme activity in chyme and mucosa are significant, $p \le 0.001$ (Fig. 3). The temperature optimum of peptidase activity of the intestinal mucosa corresponds to 40°C, chyme—60°C, enteric microbiota—between 50 and 60°C. The relative enzyme activity of chyme pike over the range 0–30°C is significantly higher that it of the enteric microbiota and in the intestinal mucosa. At 0°C the relative peptidase activity of mucosa, chyme and microbiota is 12, 80 and 18%, respectively. The level of HA of intestinal chyme, mucosa and enteric microbiota in pike at 20°C was 2.66 ± 0.12, 0.83 ± 0.10 and 0.52 ± 0.11 μmol/(g min), respectively. The differences in the level of enzyme activity of the first two samples are statistically significant, $p \le 0.001$. The temperature optimum of chyme and enteric microbiota enzymes was at 50°C, the intestinal mucosa—at 60°C. At 0°C the relative enzyme activity of mucosa was 7, chyme—24, enteric microbiota—27% of maximum activity.

In winter, the level of CA activity of the intestinal mucosa in the burbot, which feed more actively in winter then in summer, at 20°C was 3.58 ± 0.19, chyme activity—6.57 ± 0.20, enteric microbiota—4.20 ± 0.12 μmol/(g min). At that CA activity in chyme was significantly higher then in mucosa, $p \le 0.001$ (Fig. 4). The maximum activity of mucosa and enteric microbiota enzymes was found at 50°C, chyme—at 30°C. However, in the area of 20–50°C CA values were closed. At 0°C the level of investigated peptidase activity in the case of mucosa, chyme and enteric microbiota was 15, 45 and 25%, respectively. Mucosa HA in burbot at 20°C was 1.12 ± 0.08, chyme—2.29 ± 0.21, enteric microbiota – 1.25 ± 0.07 μmol/(g · min). HA activity in chyme was significantly higher than in mucosa also, $p \le 0.001$. The temperature optimum of HA of all investigated preparations was at 50°C. Relative HA of these preparations at 0°C was 7, 16 and 15% of maximal activity, respectively.

So, in a zone of temperatures of vital activity in all investigated fishes species the CA level of mucosa and chyme is higher, than a HA. The activity of chyme peptidases is higher than it in the intestinal mucosa. The relative activity of peptidases of chyme and enteric microbiota in temperature range in a vital activity of fish is usually above than those of intestinal mucosa.

DISCUSSION

The data on the activity of casein-lytic and hemoglobin-lytic peptidases (total activity of trypsin, chymotrypsin, carboxypeptidases and leucine aminopeptidase) providing hydrolysis of food proteins in the intestines in the temperature range of a vital activity in fish, are consistent with previous results [19, 27, 35]. It will be remarked that activity of casein-lytic peptidases in the studied fish species is, as a rule, higher than the activity of hemoglobin-lytic peptidases. The degree of these differences varies depending on the fish species and the season. So, in pike CA in summer is higher than HA 15.4 times, in winter only 1.9 times. At that in burbot these differences reach 3.2 times in winter. Since proteins and polypeptides in the intestine initially hydrolyse trypsin and chymotrypsin, apparently, this phenomenon may be mainly due the differences in the ratio of trypsin-like peptidases and chymotrypsin-like peptidases. In spite of these enzymes may hydrolyse both casein and hemoglobin, it is known, that the above enzymes mainly hydrolyse the peptide bonds in the different parts of the protein molecule. The sorption of the substrate in the active center of

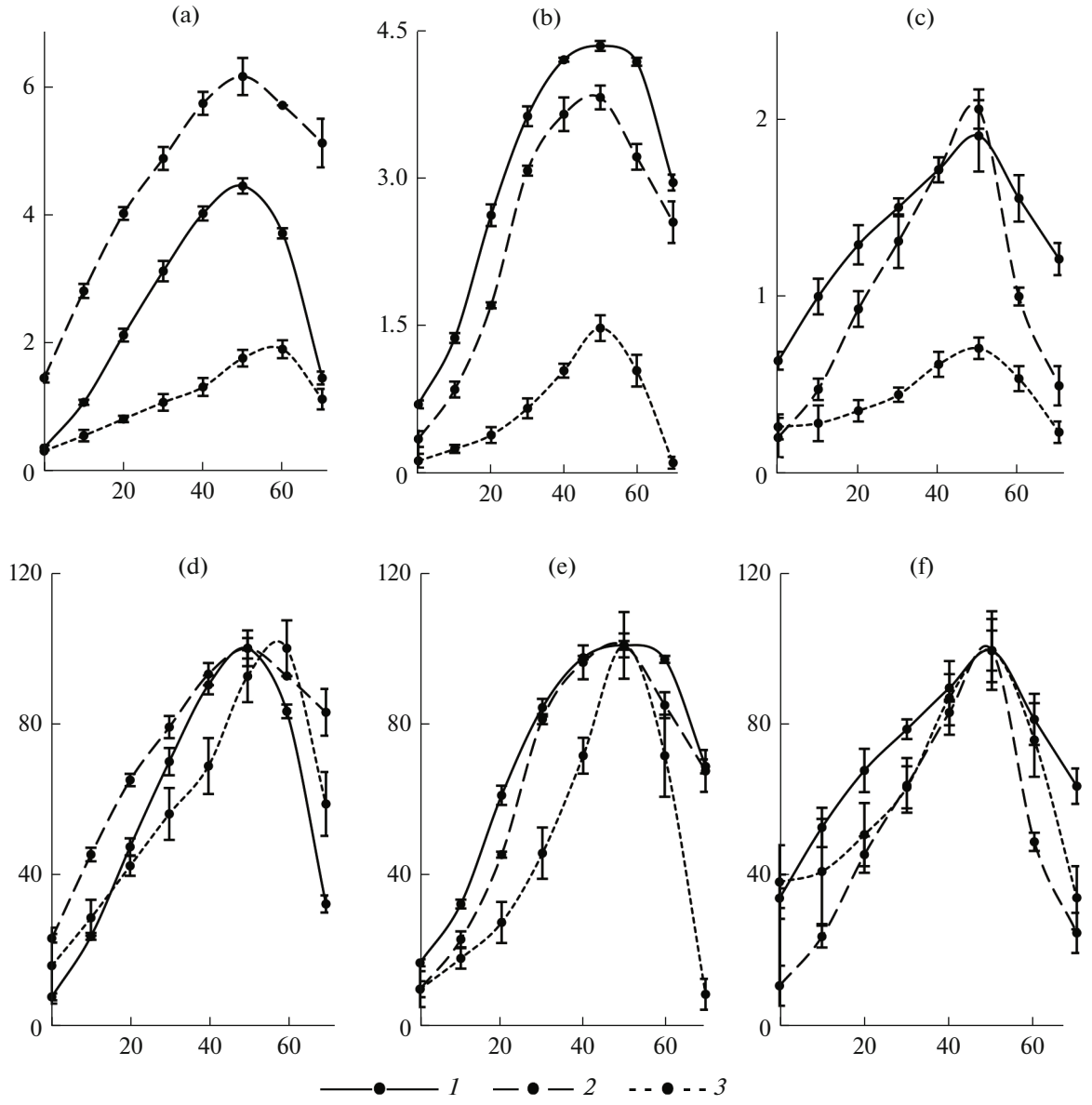

Fig. 1. Effect of the temperature on casein-lytic activity of chyme (*1*), intestinal mucosa (*2*) and enteric microbiota (*3*) peptidases in pike (a, d), zander (b, e) and perch (c, f) in summer period. Abscisses: temperature, °C. Ordinates: on (a), (b) and (c) – enzyme activity, μmol/(g · min); on (d), (e) and (f) – relative activity, % of maximum, taken for 100.

trypsin is optimal for binding the residues of aliphatic basic amino acids, namely arginine and lysine and the sorption of the substrate in the active center of chymotrypsin is optimal for binding the side chains of hydrophobic amino acid residues such as tryptophan, phenylalanine, leucine, tyrosine [10].

The activity of chyme casein-lytic peptidases in the most fish of the same species is higher than that of the intestinal mucosa. This is due to the fact that chyme, in addition to the enzymes synthesized by fish digestive system, contains the enzymes of their food objects and microbiota. Except digestive hydrolases, fish food objects contain numerous cathepsins [18, 35–41]. Unfortunately, we can not compare the activity of peptidases of enteric microbiota with that of mucosa and chyme, because of the pre-cultivation of microbiota. At the same time it is possible to compare enteric microbiota activity in fishes of various species. It was found out that casein-lytic activity of peptidases of enteric microbiota at 20°C in all studied fishes species except pike, which have a high activity of peptidases, was slightly different. The data relating to thermal characteristics of fish intestinal mucosa peptidases are similar to the results which were obtained previously with the use of synthetic substrates [1, 16, 29, 30]. Indeed, maximum of trypsin activity in Nile tilapia,

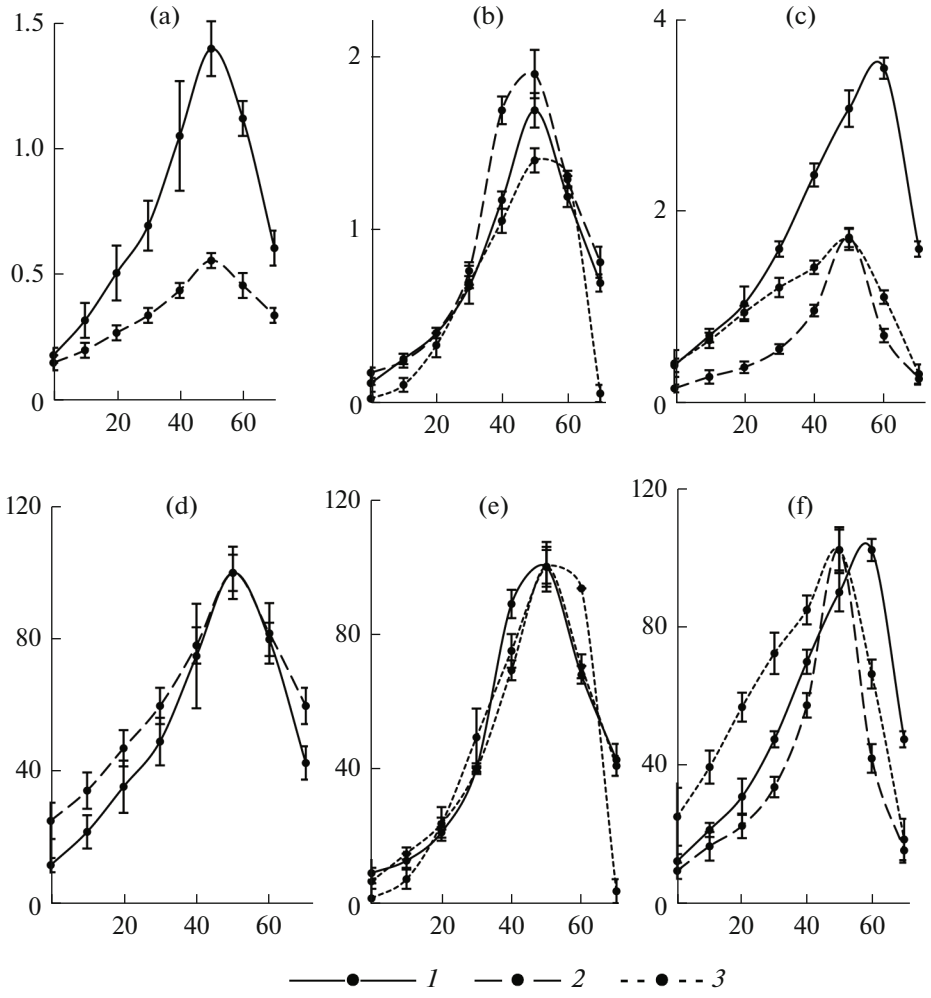

Fig. 2. Effect of the temperature on hemoglobin-lytic activity of chyme (*1*), intestinal mucosa (*2*) and enteric microbiota (*3*) peptidases in pike (a, d), zander (b, e) and perch (c, f) in summer period. Abscisses: temperature, °C. Ordinates: on (a), (b) and (c) – enzyme activity, μmol/(g·min); on (d), (e) and (f) – relative activity, % of maximum, taken for 100.

Oreochromis niloticus (L.) is observed at 50°C [3], in cod *Gadus morhua* (L.), herring menhaden *Brevoortia* spp. and mullet *Mugil* spp. are observed at 55°C [1, 4, 31]. In tambakui *Colossoma macropomum* Cuvier, 1816, Japanese anchovy *Engraulis japonicus* Temminck and Schlegel, 1846, mackerel *Scomber australasicus* Cuvier, 1832 and white croaker *Micropogohias furnieri* (Desmarest, 1823), the temperature optimum of trypsin amidase activity corresponds to 60°C [16, 29], in parona *Parona signata* (Jenyns, 1841) [30], Mayan cichlid *Cichlasoma urophthalmus* (Gunther, 1862) [7] and common snook *Centropomus undecimalis* (Bloch, 1792) [6] is 65°C. The temperature optimum of chymotrypsin in the Kamchatka salmon *Oncorhynchus mykiss* (Walbaum, 1792) correspond to 55°C (Kristjánsson, Nielsen, 1992, by: 19). Unfortunately, these data do not always correlate with the temperature of the fish habitat due to differences in the substrate used and various degree of enzyme purification. However the use of the same natural substrates (casein and hemoglobin) indicates that the temperature optimum of trypsin-like peptidases of the intestinal mucosa in burbot, Arctic faunistic complex (50°C), is lower than that of the pike, Boreal faunistic complex (60°C) [35]. The lower thermostability of burbot peptidases compared that of pike peptidases may be due to the higher flexibility of their molecules, which facilitate the functioning of enzymes at low temperatures [13].

In addition, these results confirm the weak adaptation of originally pancreatic peptidases to function in autumn-winter and spring periods of the annual cycle of fish [19, 35]. Really, the relative activity of the intestinal mucosa peptidases in the various fish species at 0°C, is, as a rule, not more than 5–20% of the maximum activity. In some fish species the activity of serine proteases, especially trypsin, in the low temperature zone can be reduced to trace values. In particular,

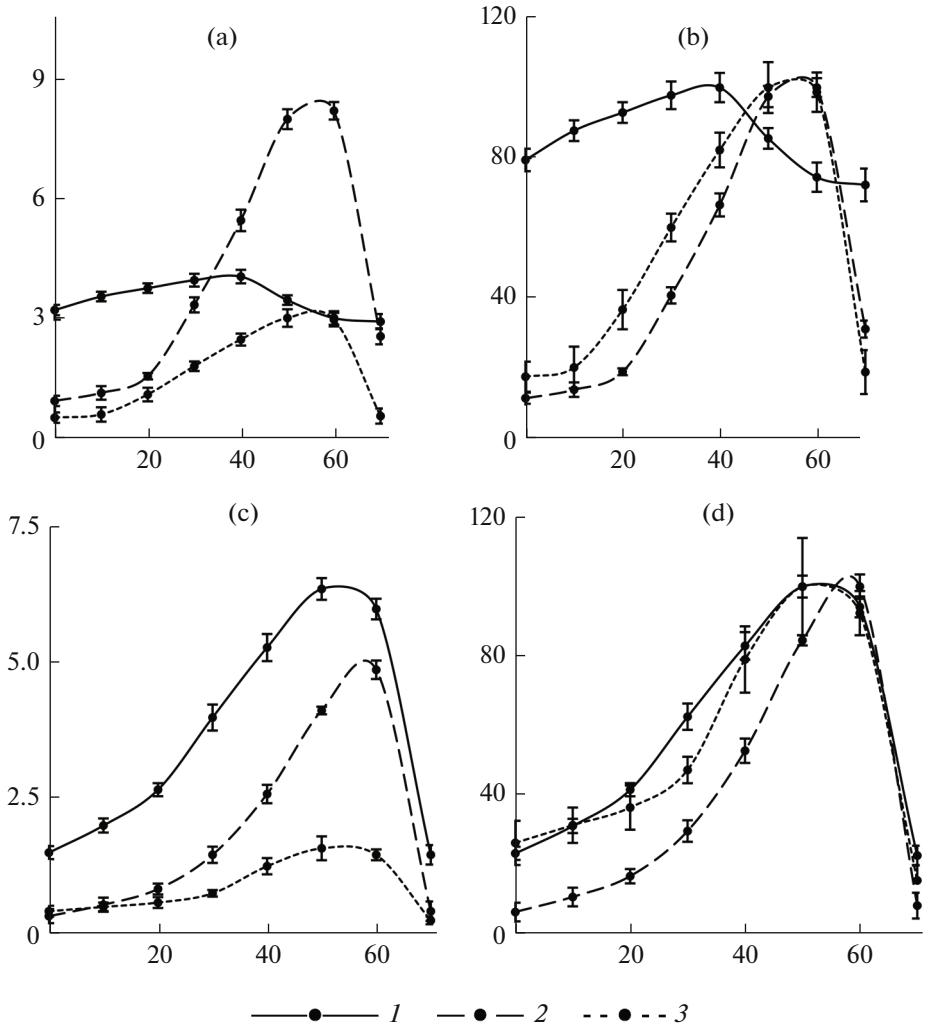

Fig. 3. Effect of the temperature on casein-lytic (a, b) and hemoglobin-lytic activity (c, d) of chyme (*1*), intestinal mucosa (*2*) and enteric microbiota (*3*) peptidases in pike in winter. Abscisses: temperature, °C. Ordinates: on (a) and (c) – enzyme activity, μmol/(g·min); on (b) and (d) – relative activity, % of maximum, taken for 100.

the turbot at 5°C retained only 0.8% of the maximum activity of trypsin [25]. At 4°C, the activity of trypsin was not detected in white grunt *Haemulon plumieri* (Lacépède, 1801) at all [26].

However, in winter in burbot and pike the relative activity of casein-lytic peptidase of chyme in a zone of low temperatures is much higher than it of intestinal mucosa enzymes (45 and 80% of the maximum activity, correspondingly). High values of relative activity of casein-lytic peptidases of the chyme in fish in a zone of low temperatures may be due to both the properties of the enzymes of fish food items, and the properties of peptidases of the associated microbiota. Temperature characteristics of enteric microbiota peptidases in most cases are similar to that of intestinal mucosa. At the same time, the relative activity of casein-lytic peptidases in a zone of low temperature can sometimes reach 30–40% of maximal activity. These results are similar to the data of the high relative activity of casein-lytic peptidases of enteric microbiota, desorbed from the tegument of cestodes and, living in the gut of pike and burbot, in a zone of low temperatures: 40–60% of maximum activity, depending on the pH and the fish species [19].

Thus, significant differences in temperature characteristics of casein- and hemoglobin-lytic peptidases of the intestinal mucosa, chyme and enteric microbiota in carnivorous boreal fish were revealed. The character of the temperature dependence of intestinal mucosa peptidases in various fish species is similar enough, while that of chyme and enteric microbiota varies largely. Relative activity of intestinal mucosa peptidases in the species studied at 0°C, is typically not more than 5–15% of maximal activity. Relative activity of chyme and enteric microbiota peptidases in a zone of low temperatures is usually above. The high-

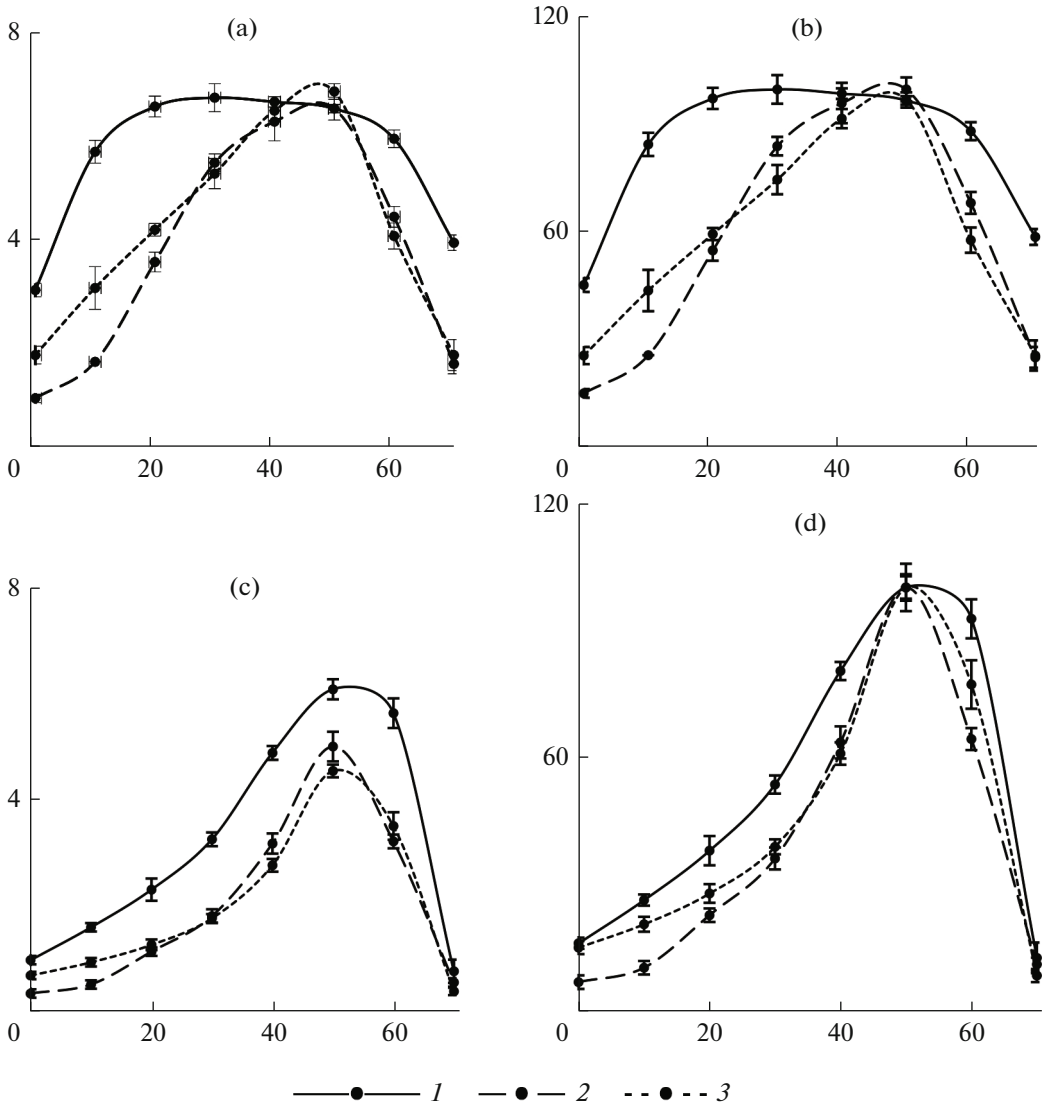

Fig. 4. Effect of the temperature on casein-lytic (a, b) and hemoglobin-lytic activity (c, d) of chyme (*1*), intestinal mucosa (*2*) and enteric microbiota (*3*) peptidases in burbot in winter. Abscisses: temperature, °C. Ordinates: on (a) and (c) – enzyme activity, μmol/(g · min); on (b) and (d) – relative activity, % of maximum, taken for 100.

est values of the relative activity of chyme casein-lytic peptidases at 0°C (45 and 80% for burbot and pike, respectively) revealed in the winter provide experimental support for the hypothesis that the lack of adaptable changes in fish intestinal peptidases can be compensated by adaptive properties of the enzymes of enteric microbiota and fish prey. So, the trophic transfer efficiency depends not only on the functional characteristics of enzyme systems of fish, but also on the functional characteristics of enzyme systems of associated and enteric microbiota.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interests. The authors declare that they have no conflict of interest.

Statement on the welfare of animals. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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