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Separate and Combined Effects of Cadmium, Temperature, and pH on Digestive Enzymes in Three Freshwater Teleosts

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Enzyme activities are sensitive to fluctuations in temperature and pH (Ugolev, Kuz'mina 1993). Pollutants can also affect enzyme function (Gill et al. 1991, Golovanova et al. 1994, Kuz'mina, Golovanova 1997), but the effects are usually studied at standard conditions (20°C and pH 7.4). However, the toxicity of pollutants is dependent on temperature, pH, nature of pollutant and fish species (Gardner, Yevich 1969, Rehwoldt et al. 1972, Cusimano et al. 1986, Nakagava, Ishio 1989). Along with mercury, zinc, and copper, cadmium is one of the most dangerous pollutants. Cadmium is a common pollutant from electroplating, smelting and refining industries around the world. Although cadmium concentration in fresh water is usually less than 0.01 mg/L, sometimes reaching 10 mg/L or greater due to industrial pollution, its concentration in fish tissues can be 10–1000 times greater than that in surrounding water as a result of bioaccumulation (Yang, Chen 1996).

The present study examined the separate and combined effects of cadmium, temperature, and pH on *in vitro* hydrolysis of carbohydrates and proteins by intestinal hydrolases from three freshwater teleosts that have different feeding strategies [bream (omnivore), and zander and perch (carnivores)].

MATERIALS AND METHODS

Bream (*Abramis brama* L.) (weight 760±24 g), perch (*Perca fluviatilis* L.), (52±3 g) and zander (*Stizostedion lucioperca* L.) (1600±40 g) were caught by trawling in Rybinsk reservoir (Russia, 57°N, 36°E). Within one hour after capture, the fish were transported to laboratory, where they were killed immediately by a blow on the head. The intestine was removed and rinsed by cold (2-4° C) Ringers for ectotherms (109 mM NaCl, 1.9 mM KCl, 1.1 mM $CaCl_2$, 1.2 mM $NaHCO_3$, pH 7.4). The mucosa from mid intestine was removed by scraping, weighted, suspended in 9 volumes of cold Ringers solution and homogenized with a glass homogenizer for 3 min. The homogenates were frozen (-20° C) and analyzed within 15 hours (preliminary studies showed that freezing did not affect enzyme activity).

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The homogenate solutions were used to study enzyme activity in a factorial arrangement of 18 experimental conditions. These included combinations of 2 Cd concentrations (0 and 50 mg Cd/L, prepared using $CdSO_4$ dissolved in Ringers), 3 temperatures (0°, 10° and 20° C) and 3 pH values (5.0, 7.4 and 8.5, pH was adjusted by adding HCl or NaOH to the Ringers). Homogenates from individual fish were diluted 2 to 4-fold with the various combinations of Ringers for assays of amylolytic activity and 10-fold for measurement of proteolytic activity. The diluted homogenates were mixed 1:1 with the 18 experimental solutions and incubated for 60 min. Aliquots of the resulted solutions 0.5 ml were incubated for 30 min with 0.5 ml of substrate (casein (1%) for protease assay or soluble starch (18 g/L) for amylase assay). The substrate solutions were prepared with the same combinations of Cd, temperature, and pH.

Proteolytic activity was measured by the increase in tyrosine using the modified method of Anson (1938). Amylolytic activity was determined by the increase in hexoses using the method of Nelson (1944) as modified by Ugolev and Iezuitova (1969). The concentrations of tyrosine and hexoses were determined colorimetrically at 597 nm and compared to tyrosine (1 mM) and D-glucose (2 mM) standards. Enzyme activity was calculated as a rate of substrate hydrolysis and expressed as μmole/g wet tissue per min.

The PROC GLM procedure of SAS (Statistical Analysis System, version 7.1, Cary NC) was used to determine if the main effects and their interactions influenced enzyme activity. LSD procedure was used to identify specific differences when significant mean effects were detected. For all comparisons, $P<0.05$ was accepted as the critical value.

RESULTS AND DISCUSSION

Enzyme activity differed significantly among the three fish species. Corresponding with differences in natural feeding habits, protease activity was highest in carnivorous zander, intermediate in perch, and lowest in omnivorous bream. A contrasting pattern was observed for amylase activity.

The effect of pH on protease and amylase activities was significant for all three species studied (Table 1; Figs 1 and 2). The lowest activity was observed at pH 5.0, whereas enzyme activity at pH 7.4 and 8.5 was dependent on an interaction between temperature and pH. The pH range studied (5 to 8.5) corresponds with the wide variation measured in fish intestine. Indeed, pH values in fish gut can reversibly change under acidic feed or water (Amerio et al. 1991), which can occur with natural (snow-water and swamping) or industrial acidification. The significant decrease in hydrolase activities at pH 5 corroborates previous findings (Ugolev, Kuz'mina 1993). It is interesting that the decline in pH from 7.4 to 5.0 (at 20°C) reduced proteolytic activity more than amylolytic activity for the carnivorous zander and perch (6.6 and 3.4-fold for proteolytic activity and 2 and 1.8-fold for amylolytic activity, respectively). In the omnivorous bream, amylolytic activity decreased 3.8 times and proteolytic activity only 1.2 times.

The varying sensitivity of digestive enzymes to pH conditions may be associated with evolutionary patterns for fish from different ecological groups, as is known for different temperature optimum among species.

Amylolytic activity at pH 7.4 was directly related to temperature for all three species. The increase in carbohydrate hydrolysis from 0 to 20°C was 3.8 times for bream, but only 1.8 times for zander and perch. These findings coincide with our previous results for the influence of temperature on α-amylase function for the same fish species. Proteolytic activity at pH 7.4 was also directly related to temperature with increases of 2.1, 3.3, and 4.0-fold between 0°C to 20°C for bream, perch and zander. Interestingly, the increase in temperature from 0 to 20°C (at pH 7.4) elevated proteolytic activity more than amylolytic activity in perch and zander only. This suggests that carbohydrase activity is less temperature-sensitive. This may be particularly true for obligatory and facultative predators that continue to feed even at 0°C compared to benthophagous bream, which stop feeding at 7°C (Ugolev, Kuz'mina 1993). The increase in amylase activity between 0° and 20°C varied among the pH conditions. It is important to note that temperature values used in this study were close to environmental temperatures for boreal fish: nearly 0°C in winter, 10°C in spring or autumn, and 20°C in summer.

For all three species, lowering the pH to 5.0 significantly reduced amylolytic and proteolytic activities at all temperatures. In bream, maximal amylolytic activity was measured at pH 7.4, but at pH 8.5 in zander and perch. In contrast, only in bream did increasing the pH to 8.5 further enhance proteolytic activity at all temperatures. For all three species, the combined effect of lowering both temperature and pH exceeded the sum of the separate effects on both proteolytic and amylolytic activities. This corresponds with previous studies that demonstrated the combined influences of temperature and pH on the activities of fish digestive enzymes exceed those for the sum of the separate effects (Ugolev, Kuz'mina 1993). The maximal decrease in the enzyme activity for all three species occurred with combined effects of 0°C and pH 5.0 when compared to pH 7.4 and 20°C.

Presence of cadmium led to a 39% decrease in proteolytic activity in perch only when all three species were considered at 20°C and pH 8.5. In perch, the combined influence of temperature and cadmium decreased proteolytic activity by 1.9-fold at 10°C and 5.3-fold at 0°C (pH 8.5). Although proteolytic activity was the lowest under the combined influence of cadmium, 0°C and pH 5.0, the interaction among temperature, pH, and cadmium was not significant for any of the fish.

Results of the present study indicate a greater sensitivity of carbohydrase activity to cadmium at 10 and 20°C in alkaline conditions for carnivorous fish, and at a neutral pH for omnivorous fish. Significant negative effect of cadmium was detected in bream at pH 7.4 and 10°C. At pH 8.5, this effect was significant in perch at 20°C and zander at 10° and 20°C. However, an interaction between

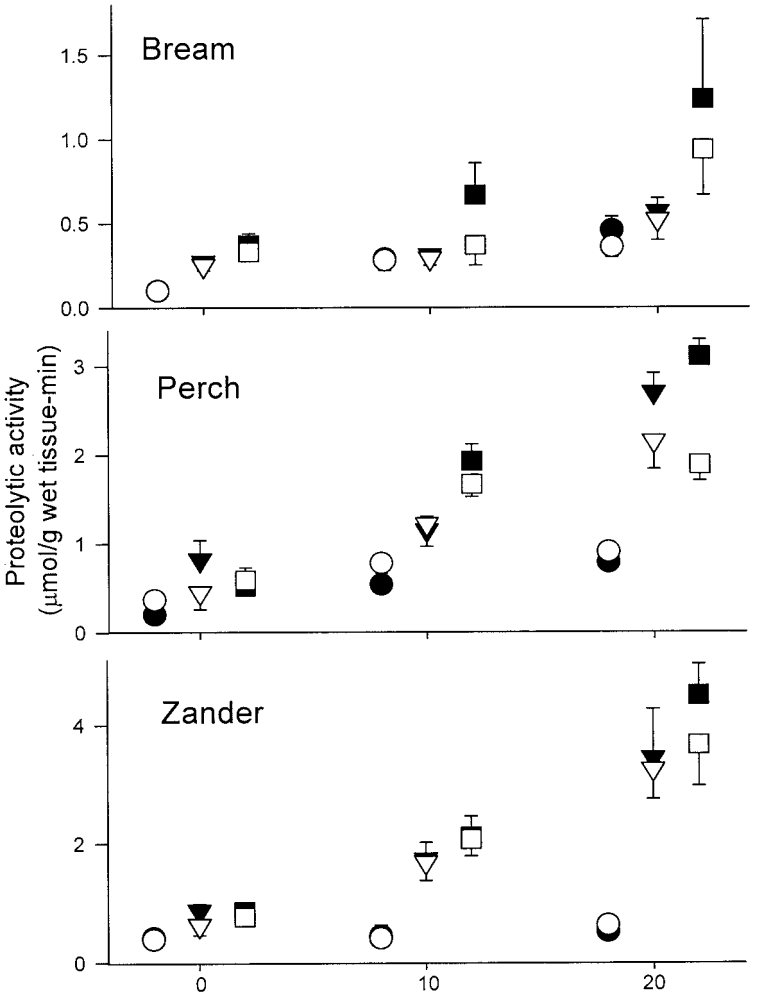

Figure 1. Proteolytic activity as a function of pH, temperature, and cadmium concentration (black symbols: 0 mg Cd/L, white symbols: 50 mg Cd/L). Mean ± SEM. Results from analysis of variance (*P* values):

Species	pH	t	pH*t	Cd	pH*Cd	t*Cd	pH*Cd*t
Bream	***0.0109***	***0.0001***	*0.4023*	*0.2107*	*0.6870*	*0.8495*	*0.9894*
Perch	***< 0.0001***	***< 0.0001***	***< 0.0001***	***0.0308***	***0.0101***	***0.0154***	*0.1129*
Zander	***< 0.0001***	***< 0.0001***	***< 0.0001***	*0.3884*	*0.7924*	*0.8404*	*0.9209*

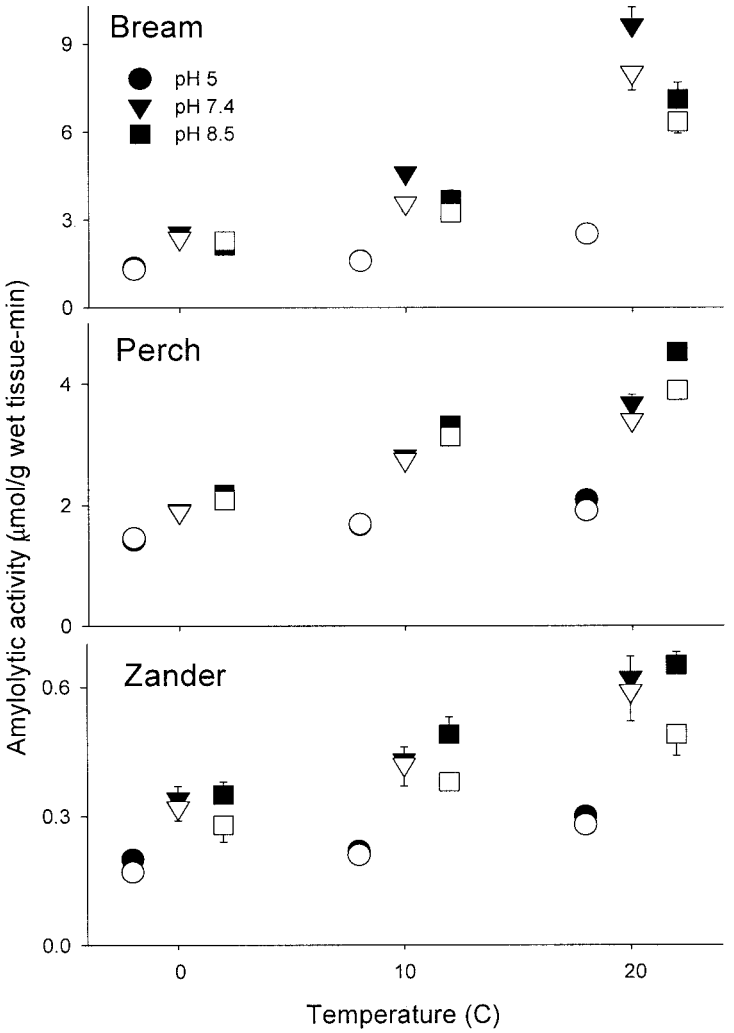

Figure 2. Amylolytic activity as a function of pH, temperature, and cadmium concentration (black symbols: 0 mg Cd/L, white symbols: 50 mg Cd/L). Mean± SEM. Results from analysis of variance (*P* values):

Species	pH	t	pH*t	Cd	pH*Cd	t*Cd	pH*Cd*t
Bream	***< 0.0001***	***< 0.0001***	***< 0.0001***	***0.0022***	*0.0711*	*0.0652*	*0.4803*
Perch	***< 0.0001***	***< 0.0001***	***< 0.0001***	***0.0003***	***0.0171***	***0.0020***	*0.6276*
Zander	***< 0.0001***	***< 0.0001***	***0.0191***	***0.0179***	*0.1055*	*0.7043*	*0.8925*

temperature and cadmium was detected only in perch. The interaction among temperature, pH, and cadmium on amylolytic activity was not significant for any of the fish.

The present data for the separate effects of cadmium, temperature, and pH on proteolytic and amylolytic activities in fish mucosa correspond closely with results of previous studies (Ugolev, Kuz'mina 1993, Golovanova et al. 1994, Kuz'mina, Golovanova 1997). Although environmental cadmium concentrations of 50 mg/L occur only in extreme situations, this concentration (but not 0.5-25 mg/L) decreased *in vitro* digestive enzyme activity in some freshwater teleosts (Golovanova et al. 1994, 1999). Our *in vitro* results also demonstrate the cadmium-induced decreases in digestive enzyme activities are magnified at higher temperatures and neutral to alkaline pH. These findings correspond with the higher cadmium toxicity for fundulus *(Fundulus heteroclitus)* at 20°C compared to 5°C, and for fundulus and medaca *(Oryzias latipes)* larvae (but not eggs) at higher pH (Gardner, Yevich 1969, Nakagava, Ishio 1989). The already low rates of protein and carbohydrate hydrolysis under low temperature and acidic conditions probably prevented detection of an effect of cadmium in the present study. The adverse effects of cadmium on protein and carbohydrate hydrolysis can potentially be significant at 10-20°C and optimal pH. This is critical since metabolic rates and dietary inputs for most species are highest under these conditions and the need for digestive enzymes is greatest. It should be noted that this study was focused on acute responses. There is a need to determine the impact of chronic exposure to cadmium and other pollutants in combination with different temperature and pH conditions on enzyme activities.

Thus, the study revealed that in acute conditions the interaction among temperature, pH, and cadmium on enzyme activity was not significant for any of the fish studied. The greatest effect on enzyme activity had the interaction of temperature and pH with the greatest decrease in activity found at low temperatures and pH 5.0.

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