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# The role of dipeptide on fish growth and digestive enzyme activity modulation in common carp (*Cyprinus carpio* L.)*

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**Meat of the common carp is an important protein source commonly used in the human diet. To increase profitability of common carp production it is essential to formulate new, innovative feeds and feeding regimes. These steps will have a beneficial effect on the reduction of the final product price thanks to intensification of the production cycle and, therefore, increase in the final body mass. The aim of the research was to evaluate the effects of feeding diets containing wheat gluten supplemented with the Lysine-Glycine dipeptide or its free forms on digestive enzyme activity, growth and economics of the common carp aquaculture. To reduce costs and increase the growth rate, fish were fed for 28 days three formulated diets containing wheat gluten supplemented with the Lys-Gly dipeptide (PP), free lysine and glycine (AA), and one diet without lysine supplementation (CON). The fourth group was fed the commercial Aglo Norse diet (AN).**

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**The highest final survival rate and body weight were recorded in fish fed AN. Analysis of digestive enzyme activities revealed that in carp fed AN the activities of trypsin, alkaline phosphatase and leucine aminopeptidase were significantly higher than in fish fed the gluten-containing diet. The highest amylase activity was observed in the PP group, while the highest lipase activity was recorded in the AA group.**

**The AN diet had a beneficial influence on the survival rate and final body weight of fish, while simultaneously increasing the economic profit. In turn, supplementation of the diet based on wheat gluten with the Lys-Gly dipeptide improved digestive enzyme activities (except for lipase) compared to feeding lysine-deficient feeds or supplemented with free amino acids.**

**The use of commercial feed formulated based on fishmeal in common carp breeding results in a 3-fold increase in fish growth rate compared to the experimental diets. The faster growth has a beneficial economic effect due to the increase in final weight of fish obtained within a shorter production cycle.**

**KEY WORDS: amino acids / common carp / digestive enzyme activity / dipeptides / wheat gluten**

Aquaculture is the fastest growing animal food production sector in the world. In Europe most carp aquacultures are located in Poland, the Czech Republic, Hungary, Germany, Ukraine and Russia. This species is also bred e.g. in China and Israel. The global production reaches 3 million tons. In Poland over 90% of pond aquaculture production is connected with two species: the common carp (*Cyprinus carpio* L.) and the rainbow trout (*Oncorhynchus mykiss* Walbaum), with the production volume of the former species reaching up to 15000 tons per year. It accounts for about 60% of total aquaculture production and it is traditionally sold almost completely during the Christmas season [Kaczkowski and Grabowska 2016]. Carp in Poland is bred in earthen ponds in semi-intensive systems with grain feeding supplementation, which allows to obtain a commercial size fish (1500-2000 g) within 2-3 years. Introduction of innovative feeds could shorten the production cycle and thus reduce the price of the final product. The increase in aquaculture production of fish requires high content of fish meal, which is one of the main components of fish feeds. Due to growing costs of fish meal it is essential to search for new, alternative protein sources [Hardy 2010]. For economic reasons introduction of diets based on alternative protein sources may improve profitability of semi-natural carp breeding. According to Turkowski and Lirski [2010], the nutrition cost accounts for as much as 20% of total production costs. Next to labour costs, the cost of fish nutrition is the largest expense in aquaculture of this species. As pointed out by Turkowski and Lirski [2010], the economic situation of carp farms is not easy because of the serious burdens on their budgets, connected with fish survival, diseases, predation and water use cost. The introduction of innovative feds, especially in the 1st and 2nd year of production, may increase fish survival rates, currently amounting to about 36-38%, and thus will improve profitability of carp aquaculture. Physiology of nutrient digestion during ontogenesis is closely related to the morphology of the digestive system and may indicate the digestive potential of fish [Kamaszewski *et al.* 2014a]. Therefore, the activity of digestive enzymes may be a very effective indicator of fish larvae development [Zambonino Infante and Cahu

2001] and nutritional condition of fish [Kamaszewski *et al.* 2010, Kamaszewski *et al.* 2014b].

Extensive research has been carried out concerning the use of soybean meal, casein, wheat gluten and other products as alternative sources of protein in diets for various fish species [Kamaszewski *et al.* 2010, Ostaszewska *et al.* 2010, Jalili *et al.* 2013]. Wheat gluten commonly used in aquaculture nutrition is one of the most promising components, which has an additional advantage - it is a by-product of starch extraction from wheat. Wheat gluten is characterised by high contents of readily digestible protein abundant in glutamine and it is free from antinutrients [Apper-Bossard *et al.* 2013]. On the other hand, gluten is deficient in arginine, tryptophan and particularly in lysine – the main amino acid limiting fish growth. Therefore, in formulation of gluten-based fish diets lysine supplementation is necessary [Apper-Bossard *et al.* 2013].

No data are available on the effects of dipeptide supplementation on digestive enzyme activity in agastric fishes such as the common carp (*Cyprinus carpio* L.). The aim of this study was to investigate the effect of feeding diets containing wheat gluten supplemented with dipeptides or free amino acids on digestive enzyme activity, growth and economics of common carp aquaculture.

## Material and methods

### Animals

This protocol was evaluated and approved by the 3rd Warsaw Local Ethics Committee for Animal Experimentation at the Warsaw University of Life Sciences (No. 28/2006). The experiment was carried out at the Department of Ichthyobiology, Fisheries and Aquaculture Biotechnology, the Warsaw University of Life Sciences – SGGW. Juveniles of the common carp (*Cyprinus carpio* L.) at the age of 1 month and average body weight of 0.07±0.02 g (measured with a digital caliper; Topex, Warsaw, Poland) were obtained from the hatchery of the Inland Fisheries Institute in Olsztyn, the Experimental Fish Farm in Żabieniec.

### Water quality analysis and experimental design

The fish were reared in the recirculating aquaculture systems (RAS) in 16 tanks of 20 L, at a stocking density of 2.5 fish $L^{-1}$ (800 fish in the entire experiment; for each group 4 tanks×50 fish). Water parameters during the experiment were as follows: average water temperature was 20.9±0.9°C, pH 6.9±0.1, dissolved oxygen concentration 8.07±0.34 mg $L^{-1}$, the level of $NH_4^+$ was 0.03±0.01 mg $L^{-1}$ and the concentration of $PO_4^{3-}$ was 0.2±0.17 mg $L^{-1}$. The 12-hour photoperiod was maintained throughout the experiment.

The experiment lasted 28 days. The fish were divided into 4 groups, with 4 replicates each. The fish were fed 3 formulated diets containing wheat gluten supplemented with Lys-Gly dipeptide (diet PP), free lysine and glycine (diet AA) or without amino acid

supplementation (diet CON; the control). Chemical compositions of diets are shown in Table 1. The fourth group of fish was fed the commercial diet Aglo Norse (Larvae Feed Ewos, Bergen, Norway; diet AN). All the experimental diets were formulated in the Aquaculture Laboratory, the Ohio State University, USA. During the experiment the fish were fed every 2 hours from 08:00 to 20:00. In the first two weeks the daily feeding rate was equal to 10% of fish body weight and then it was reduced to 8%.

**Table 1.** Ingredients and nutrient composition of diets

| Item | Content of component in the feed (g $kg^{-1}$) | | |
|---|---|---|---|
| | CON diet | PP diet | AA diet |
| Ingredients | | | |
| fish meal | 124 | 124 | 124 |
| wheat gluten* | 370 | 370 | 370 |
| wheat meal | 171.8 | 171.8 | 171.8 |
| fish oil | 50 | 50 | 50 |
| lecithin | 150 | 150 | 150 |
| mineral mix | 30 | 30 | 30 |
| vitamin mix | 40 | 40 | 40 |
| lysine-glycine** | - | 29 | - |
| lysine*** | - | - | 18 |
| glycine*** | 11 | - | 11 |
| glutamate**** | 18 | - | - |
| cysteine***** | 2 | 2 | 2 |
| arginine | 65 | 65 | 65 |
| methionine | 3.3 | 3.3 | 3.3 |
| threonine | 2.9 | 2.9 | 2.9 |
| ca-mono p | 15 | 15 | 15 |
| vitamin c | 15 | 15 | 15 |
| Nutrient composition (g $kg^{-1}$) (in dry weight basis) | | | |
| crude lipid | 60 | 60 | 60 |
| ash | 70 | 70 | 70 |
| moisture | 80 | 80 | 80 |
| gross energy (MJ $kg^{-1}$) | 196 | 196 | 196 |

*MP Biomedicals, Solon, OH, USA; **MP Biomedicals; ***Bachem, New York, NY, USA; ****Hughes [1985] – non-toxic level of glutamate; *****Hara [2006] – most potent olfactory stimulating amino acid.
CON – diets containing wheat gluten without amino acid supplementation; PP – diets containing wheat gluten supplemented with Lys-Gly dipeptide; AA – diets containing wheat gluten supplemented with free lysine and glycine.

**Sample collection and chemical analysis**

On the last day of rearing before the first feeding all fish were weighed accurate to 1 mg and the recorded data were used to estimate the survival rate. Afterwards 5 fish from each tank (20 fish from each group) were collected randomly and euthanised with MS-222 (1:5000 MS-222, pH 7.5 adjusted with $NaHCO_3$; Sigma, Steinheim, Germany). Then digestive tracts were isolated from 5 fish from each tank, frozen in liquid nitrogen and stored at -80°C.

At the end of the experiment the survival rate, specific growth rate (SGR), protein efficiency ratio (PER) and feed conversion ratio (FCR) were calculated according to the standard procedures and the following formulas [Ostaszewska *et al.* 2011]:

SGR %/day = 100×[ln(final BW) − ln(initial BW)]/days, where: BW − body weight;

FCR − dry feed intake (g)/weight gain (g);
PER − body weight gain (g)/protein fed (g).

The survival rate was monitored daily and calculated as the average percentage from 4 tanks (n=4).

**Enzyme activity analysis**

To evaluate the digestive enzyme activities assay of: amylase (EC 3.2.1.1), lipase (EC 3.1.1.3), trypsin (EC 3.4.21.4), alkaline phosphatase (ALP; EC 3.1.3.1), leucine aminopeptidase (LAP; EC 3.4.11.1) and protein levels, samples of digestive tracts were homogenised in buffers according to the methods described below and then centrifuged at 4ºC for 15 min. at 15000×g. Enzyme activity was analysed according to the procedures described for amylase [Foo and Bais, 1998], lipase [Winkler and Stuckman 1979], trypsin [Erlanger *et al.* 1961] and ALP [Wenger *et al.* 1984] and LAP [Nagel *et al.* 1964]. Protein content was evaluated according to Lowry *et al.* [1951]. All analyses of enzyme activities were performed in 5 replicates using the kinetic method at 25°C. Enzyme activities were expressed as the number of micromoles of the reaction product per 1 minute and calculated for 1 mg of protein (U/mg protein). Absorbance was measured using a M501 Camspec spectrophotometer (Camspec Ltd., Sawston, United Kingdom).

**Statistical analysis**

The data (survival rate, growth rate, PER, FCR and activities of digestive enzymes) were assessed for normality using a Shapiro-Wilk test and submitted to one-way ANOVA and post-hoc Tukey's test ($p \leq 0.05$). These computations were performed using Statistica 12.0 software (StatSoft. Inc., Tulsa, OK, USA)

## Results and discussion

The use of dipeptides in fish nutrition has been studied for the last decade. Larvae and juveniles of teleost fish absorb amino acids and dipeptides derived from protein and peptide digestion in the alimentary tract. According to Dabrowski *et al.* [2005], dipeptides may be used to supplement indispensable amino acids (IDAA) in early developmental stages of vertebrates. It was reported that in some fish species selected amino acids such as arginine and phenylalanine may be used as dipeptides, but no difference was observed between utilisation of dipeptides compared to free amino acids [Kim *et al.* 2012]. On the other hand, supplementation of diets with the Lys-Gly dipeptide improved growth rate, whole body amino acid composition and digestive

tract morphology in many fish species [Ostaszewska *et al.* 2010, Ostaszewska *et al.* 2013]. Better utilisation of lysine applied in Lys-Gly dipeptide compared to the free amino acid is related to the activity of the intestinal oligopeptide transporter PepT1 [Ostaszewska *et al.* 2009]. Therefore, studies were undertaken on the applicability of the Lys-Gly dipeptide in lysine supplementation of diets based on lysine-deficient wheat gluten.

The statistical analysis at the end of the experiment demonstrated a significantly higher survival rate of fish from the AN group compared to those fed formulated diets based on wheat gluten (Tab. 2). Similarly, fish fed the AN diet showed significantly higher body weight compared to the other groups, with no significant differences found between fish fed gluten-containing diets (Tab. 2). The highest PER and the lowest FCR values were recorded for fish fed the AN diet (Tab. 2). Among fish fed the experimental wheat gluten-based diets the greatest body weight and PER and the lowest FCR were found in the PP group supplemented with the Lys-Gly dipeptide (Tab. 2).

**Table 2.** Survival, body weight, SGR, PER and FCR of carp after 28 days of experimental feeding (mean with their SD, n = 4 tanks)

| Parameter | Feeding group | | | |
|---|---|---|---|---|
| | CON | PP | AA | AN |
| Survival (%) | $85.0^{b}$ (5.8) | $85.3^{b}$ (1.2) | $83.3^{b}$ (5.8) | $99.5^{a}$ (1.0) |
| Body weight (g) | $0.19^{b}$ (0.06) | $0.23^{b}$ (0.06) | $0.22^{b}$ (0.06) | $0.61^{a}$ (0.28) |
| SGR (%/day) | $3.73^{c}$ (0.22) | $4.32^{b}$ (0.22) | $4.23^{b}$ (0.19) | $7,83^{a}$ (0.29) |
| FCR (g) | $2.02^{a}$ (0.15) | $1.71^{b}$ (0.16) | $1.94^{a}$ (0.15) | $0.63^{c}$ (0.09) |
| PER (g /g) | $1.2^{c}$ (0.13) | $1.41^{b}$ (0.15) | $1.24^{bc}$ (0.08) | $2.99^{a}$ (0.15) |

$^{abc}$Within rows means bearing different superscripts differ significantly at $p \leq 0.05$.
SGR – specific growth rate; FCR – feed conversion ratio; PER – protein efficiency ratio.
CON – diets containing wheat gluten without amino acid supplementation; PP – diets containing wheat gluten supplemented with Lys-Gly dipeptide; AA – diets containing wheat gluten supplemented with free lysine and glycine; AN – commercial diet.

Ostaszewska *et al.* [2010] reported that feeding diets containing wheat gluten supplemented with the Lys-Gly dipeptide and free lysine and glycine resulted in good survival rates, growth rates, appropriate digestive tract morphology, homeostasis of the intestinal epithelium and expression of the oligopeptide transporter PepT1 in the intestinal brush border of common carp. Similar results concerning the effects of fish meal substitution with wheat gluten in diets on growth and survival rates of fish were obtained by Jalili *et al.* [2013].

In omnivorous and herbivorous fish the activity of amylase is higher compared to predatory species, which is related to the higher content of dietary carbohydrates [de la Parra *et al.* 2007] or lipids [Kamaszewski *et al.* 2010]. Similarly, in the present study fish fed the AN diet (feed with the highest lipid content) showed a higher amylase activity compared to those fed the AA and CON diets (Tab. 3). However, the highest

**Table 3.** Activities of digestive enzymes in common carp after 28 days of experimental feeding (mean with their SD, n = 20)

| Enzymes (U /mg protein) | Feeding group | | | |
|---|---|---|---|---|
| | CON | PP | AA | AN |
| Amylase | $23.71^{b}$ (3.38) | $67.83^{a}$ (9.73) | $26.90^{b}$ (7.59) | $31.19^{b}$ (1.73) |
| Lipase | $0.14^{b}$ (0.04) | $0.08^{b}$ (0.02) | $0.69^{a}$ (0.26) | $0.08^{b}$ (0.02) |
| Trypsin | $0.30^{c}$ (0.04) | $3.21^{b}$ (0.84) | $0.34^{c}$ (0.10) | $4.50^{a}$ (0.48) |
| ALP | $4.68^{c}$ (0.57) | $20.15^{b}$ (4.87) | $4.97^{c}$ (1.04) | $28.51^{a}$ (3.05) |
| LAP | $0.23^{c}$ (0.05) | $0.83^{b}$ (0.16) | $0.38^{c}$ (0.01) | $1.11^{a}$ (0.13) |

$^{abc}$Within rows means bearing different superscripts differ significantly at $p \leq 0.05$. CON – diets containing wheat gluten without amino acid supplementation; PP – diets containing wheat gluten supplemented with Lys-Gly dipeptide; AA – diets containing wheat gluten supplemented with free lysine and glycine; AN – commercial diet.

amylase activity was observed in the group fed with a low lipid PP feed (Tab. 3). This probably resulted from the enzymatic adaptation to feed composition [Zambonino Infante and Cahu 1994].

The optimum lipid content in diets of various fish species has not been definitely determined. The results obtained by Buchet *et al.* [2000] revealed that lipase activity in fish was stimulated by an increase in dietary lipid content, but only to a certain level. The highest lipase activity was detected in carp fed the AA diet, while it was low in fish fed high lipid AN and PP diets (Tab. 3), which confirms a non-linear relationship between dietary lipid content and lipolytic activity. The lipase activity in fish from the PP, CON and AN groups was similar and did not differ significantly (Tab. 3), which may indicate that the activity of this enzyme is almost constant and depends on genetic rather than dietary factors [Kamaszewski *et al.* 2014a]. Regulatory mechanisms and dietary influence on lipolytic activity in fish certainly require further studies.

Trypsin is probably a key proteolytic enzyme in many fish species [Manchado *et al.* 2008]. Various authors emphasise the fact that activity of alkaline proteases expressed as trypsin activity depends on dietary and environmental factors [Napora-Rutkowski *et al.* 2009, Kamaszewski *et al.* 2010, Kamaszewski *et al.* 2014b]. Therefore, it is assumed that the proteolytic activity of trypsin may be a useful indicator of digestive ability in fish [Okan Kamaci *et al.* 2010]. In the present study the highest trypsin activity was observed in carp fed the AN diet (Tab. 3). This commercial diet showed a higher protein content compared to the experimental feeds. This relationship confirms the results obtained by Eusebio and Coloso [2002], who reported that in the Asian sea bass (*Lates calcifer* Bloch) trypsin activity increased with an increase of dietary protein levels. High trypsin activity in fish fed PP among fish fed wheat gluten based diets also confirms that secretion of pancreatic enzymes strongly depends on the oligopeptide content in the digestive tract that stimulates CCK secretion. According to Murashita *et al.* [2015], supplementation of fish diets with oligopeptides or with certain free amino acids may stimulate cholecystokinin (CCK) secretion, which causes an increase in the exocrine pancreatic secretion of digestive enzymes such as amylase, lipase and

trypsin. This was confirmed by the results obtained by Ostaszewska *et al.* [2010], who in common carp fed with the PP diet observed the highest number of gastrin/CCK secreting cells in the intestinal epithelium, which was related to the increased secretion of pancreatic enzymes including amylase. The obtained results show that further studies are necessary to elucidate the role of dipeptides and amino acids in the stimulation of secretion of hormones regulating exocrine pancreatic activity by neuroendocrine cells.

The enzymes secreted by the intestinal brush border are involved in final food digestion to the nutrients that are ready to be absorbed. These enzymes include phosphatases responsible for hydrolysis of inorganic phosphates, transport of nutrients through the brush border membrane into the enterocytes, modification of amino acid side chains and stimulation of calcium ion absorption [López-Ramírez *et al.* 2011]. One of these enzymes, alkaline phosphatase (ALP), is commonly used as a marker of differentiation and physiological maturity of enterocytes in fish [Gisbert *et al.* 1999]. Leucine aminopeptidase (LAP) is another enzyme secreted by the intestinal brush border and it is responsible for the final digestion of peptides to absorbable amino acids. The activity of this enzyme is a marker commonly used in fish physiology to evaluate the efficiency of food digestion and nutrient absorption. An increase in the activity of this enzyme during fish ontogenesis may be a good indicator for the development and physiological maturation of the intestinal brush border [Kvale *et al.* 2007].

The highest activity of ALP and LAP was observed in fish fed the AN diet (Tab. 3), which according to Kvale *et al.* [2007] indicates well-developed enterocytes and mature digestion processes. Lower activity of both brush border enzymes was observed in fish fed diets formulated with gluten (PP, AA and CON) compared to those fed the commercial diet (Tab. 3), which may indicate that the digestive tracts of these fish were not fully mature. However, fish fed PP diet showed an almost 4-fold higher ALP activity and 2-fold greater LAP activity when compared to fish fed the AA and CON feeds. This indicates that dipeptides stimulate differentiation and maturation of the intestinal epithelium. On the other hand, low ALP and LAP activity in the AA and CON groups may have been caused by malnutrition, nutritional deficiency or physiological immaturity of enterocytes. The ALP activity is affected by phosphorylated products such as phospholipids and phosphoproteins and thus according to Zambonino Infante and Cahu [1994] this enzyme is an indicator of malnutrition in fish. These authors also observed that LAP activity is affected not only by the dietary protein content, but also by protein composition. The LAP activity was particularly low in fish fed the CON diet, which might have resulted from amino acid deficiency. This indicates that an insufficient lysine level resulting in the reduced length of intestinal folds [Ostaszewska *et al.* 2010] also reduces LAP secretion by the brush border.

The obtained results indicate that feeding common carp with the commercial diet was beneficial for the survival rate, body weight, enzymatic activity and physiological maturation of the intestinal epithelium. The results will facilitate the development

of more effective feeding regimes, being more economical as a consequence ofthe shortened production cycle. In turn, supplementation of the diet based on wheat gluten with the Lys-Gly dipeptide improved the activity of amylase, trypsin, ALP and LAP when compared to feeding lysine-deficient feeds or feeds supplemented with free amino acids. These results indicate that supplementation with the Lys-Gly dipeptide stimulates differentiation and maturation of the intestinal epithelium in juvenile common carp fed a diet containing wheat gluten as a protein source. However, compared to the results obtained in fish fed the commercial feed, the use of feeds based on wheat gluten had no positive effect on specific growth rate (SGR), survival rate or body weight, which is rather surprising in the light of research conducted on other fish species.

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