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GHRELIN EFFECTS ON THE ACTIVITIES OF DIGESTIVE ENZYMES AND GROWTH OF *LYMANTRIA DISPAR* L.

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Abstract - Ghrelin, along with several other hormones, has significant effects on appetite and growth in humans and animals. The aim of our study was to examine changes in relative growth rates, α- and β-glucosidase activities and endocrine cell size in the midgut of 4th instar caterpillars of the pest insect *Lymantria dispar* L. after ghrelin treatment. Four subpicomolar injections of ghrelin (0.3 pmol) or physiological saline were applied every 24 h to two separate groups of fifteen caterpillars. Repeated administration of ghrelin in subpicomolar doses elevated the relative growth rate, induced α- and β-glucosidase activities and increased the size of endocrine cells. The results are the first data about ghrelin effects on relative growth rate, digestive enzyme activities and midgut endocrine cells in insects. This information supports the use of this relatively simple model system in future studies of mechanisms underlying digestion in complex organisms.

Key words: ghrelin, growth rate, midgut, glucosidase, endocrine cells

INTRODUCTION

Lymantria dispar L., a polyphagous herbivore, is the most dangerous insect pest of forest and fruit trees. Its host range is estimated at more than 500 plant species from 73 families (Liebhold et al., 1995). Populations rapidly increase, causing widespread defoliation, weakening and killing a wide range of trees. However, the exact mechanisms that drive the feeding behavior of *Lymantria dispar* L. have not yet been fully elucidated.

There are a large number of compounds in insect tissues that are identical or at least structurally very similar to vertebrate hormones involved in the regulation of feeding intensity, such as tachykininscholecystockinin (Wei et al., 2000), gastrin (Janssen at al., 2008), somatotropin-like, ACTH-like (Perić Mataruga et al., 2007), neuropeptide Y, and agouti-related protein (Wu et al., 2005). This evidence favors the idea that vertebrate and insect hormones emerged early during evolution and that they have a common origin (Nijhout, 1994). Considering the

hypothesis about evolutionary relationships among the main hormone groups, it seems that there are similarities in the mechanisms regulating feeding and energy balance and other biological activities in vertebrates and insects.

Ghrelin has emerged as the first identified circulating hunger hormone. Ghrelin and synthetic ghrelin mimetics (the growth hormone secretagogues) increase food intake and fat mass (Tschop et al., 2000; Nešić et al., 2008; Stevanović et al., 2008). Ghrelin is a 28 amino acid peptide, originally isolated from rat stomach, which acts as an endogenous ligand of the growth hormone (GH) secretagogue-receptor (GHS-R), two subtypes of which have been identified: the fully functional GHS-R1a and the biologically inactive GHS-R1b (Kojima et al., 1999).

Three types of cels have been recognized in *Lymantria dispar* midgut epithelium: columnar epithelial cells, goblet cells and endocrine cells (Perić Mataruga et al., 2006). The midgut endocrine cells are differentiated from stem cells and located among columnar cells (Zitnan et al., 1993). In some insect orders, pancreatic polypeptide (pp), somatostatin and glucagon-like immunoreactive material have been shown to be present in the midgut endocrine cells of insects besides insect neurohormones (Endo et al., 1982).

α-glucosidase (EC 3.2.1.20) catalyzes the hydrolysis of terminal, non-reducing 1,4-linked alpha-D-glucose residues while releasing alpha-D-glucose. This enzyme strongly hydrolyzes sucrose, maltose, maltodextrin and pNP-α-D-glucopyranoside. It can be found in the alimentary canal and salivary secretions of insects (Terra et al., 1996). β-glucosidase is an enzyme which acts upon β1 → 4 bonds among two glucose or glucose-substituted molecules and hydrolyzes these major bonds (β1 → 4, i.e., the disaccharide cellobiose). These enzymes are active upon a large range of components in plants. These components are involved in plant resistance to insects and insect predation by herbivores.

MATERIALS AND METHODS

Insects rearing and ghrelin treatment

Lymantria dispar egg masses were collected in an oak forest near Opovo, 30 km from Belgrade (longitude, 20825049E; latitude, 458308N; altitude, 67 m). After hatching, the caterpillars were reared on an artificial wheat germ diet (O′Dell, 1984) at 23°C, with a 16 h light : 8 h dark photoperiod. Fifteen newly molted 4th instar larvae (n=15, the same size) of *L. dispar* were used per experimental group. Each larva received subcutaneous injections halfway down the body length using an AGLA-Micrometer Syringe Outfit. During the injection, the needle was kept parallel to the cuticle to prevent any physical damage to internal tissues. The treated experimental group of larvae received 0.3 pmol of rat ghrelin (Global Peptide Services, Fort Collins, Colorado) injections in insect physiological saline (150 mM NaCl, 5 mM KCl), every 24 h for four consecutive days. The dosing regimen was calculated according to data previously reported and adjusted for the biomass of 4th instar caterpillars (Perić Mataruga et al., 2009). The control group received only physiological saline. Food consumption was taken as the daily difference between food supplied and food remaining. During the treatment, body weight, food intake, as well as frass elimination were recorded daily.

Slansky and Scriber (1985) developed specific values to quantify the growth and utilization of food by insects. The relative consumption rate (RCR) represents milligrams of ingested food per day.

RCR (g/g/day) = (weight of food eaten)/(insect weight at the start of the trial)x(time of feeding)

Enzyme assays and protein determination

α- and β -glucosidase assay: the principle of the reaction is the release of p-nitrophenol from 4-nitrophenyl α-D-glucopyranoside and *p*-nitrophenol from 4-nitrophenyl β -D-glucopyranoside which are specific substrates for α- and β-glucosidase detection (Baker, 1991). Twenty mM substrate was dissolved

in water. The substrate was added at final concentration of 2 mM to 0.2 M sodium-phosphate buffer. The incubation time was 10 min for α-glucosidase and 60 min at 30°C for β-glucosidase. The reaction was stopped by adding 1 ml of the following mixture: 0.25 M Na_2CO_3; 0.25 M $NaHCO_3$ and 1% SDS. Glucosidase activity was measured spectrophotometrically at 405 nm. The protein concentration was determined according to Bradford (1976) using bovine serum albumin as the standard. Fifteen newly molted 4th instar larvae (n=15, the same size) of *L. dispar* were used per experimental group.

Histological technique

After the caterpillars were killed, the midguts were dissected and fixed in Bouin's fixative for 24 h. The midguts were then rinsed, dehydrated and embedded in wax. Serial 3.5-μm-thick cross-sections of the midguts were stained with hematoxylin, eosin, and PAS-alcian blue. The size of the endocrine cells was expressed as the mean value of the smallest and largest diameter in μm (Panov, 1980). The significance of ghrelin effects on the observed parameters was revealed by one-way ANOVA following Fisher's Least Significant Difference (LSD) test. The acceptable P cutoff value was set at 0.05.

RESULTS

Relative growth rate

The mean growth rates of larvae treated with ghrelin and the control group are given in Fig. 1. One-way ANOVA showed a significant effect of ghrelin on the relative growth rate in the 4th larval instar of *Lymantria dispar*. The relative growth rate, measured by weighing the larvae daily, was significantly higher after ghrelin treatment than in the control group (LSD tests, $P<0.001$). Thus, on average, the larvae increased in growth by 58% after the ghrelin treatment in comparison to the control.

α- and β-glucosidase activities

The digestive enzyme activities of larvae treated with ghrelin and saline are shown in Figs. 2A, B and one-way ANOVA confirmed the effect of this hormone on the tested parameters.

β-glucosidase activity (LSD test - $P<0.05$) was significantly higher in the midgut of the ghrelin-treated larvae than in the control group (Fig. 2A). The activity of this enzyme increased in the ghrelin-treated larvae by 62.5% compared to the control group (Fig. 2B). *α*-glucosidase activity tended to increase in the ghrelin-treated larvae in comparison to the control group (Fig. 2A).

Endocrine cells characteristics

The midgut is the largest part of the digestive tract of lepidopteran larvae. It is lined with columnar cells. The endocrine cells are rarely located among the columnar epithelial cells and on the basal lamina. These cells were significantly larger (LSD test - $P<0.05$) in the midguts of the ghrelin-treated animals than in the control (Fig. 3). The size of the endocrine cells increased in ghrelin treated larvae by 30.8% compared to the control group (Fig. 2B).

DISCUSSION

Previous research has shown that ghrelin application leads to a significant increase in body mass, increased feeding, locomotor activity and shortened larval development in *Lymantria dispar* caterpillars (Perić Mataruga et al., 2009). In this paper, we present the analysis of changes in the relative growth rate and digestive enzyme activities in *Lymantria dispar,* which should help us to understand the behavioral and physiological basis of such responses. The results of this paper show alterations in the relative growth rate in 4th instar larvae treated with ghrelin. Biologically, relative growth rate is the product of relative consumption rate and the efficiency in converting ingested food, which further depends on the efficiency of utilization of digested food. We can conclude that ghrelin treatment can directly affect growth by increasing food intake, i.e. by increasing the appetite of *Lymantria dispar* caterpillars (Perić Mataruga et al., 2009).

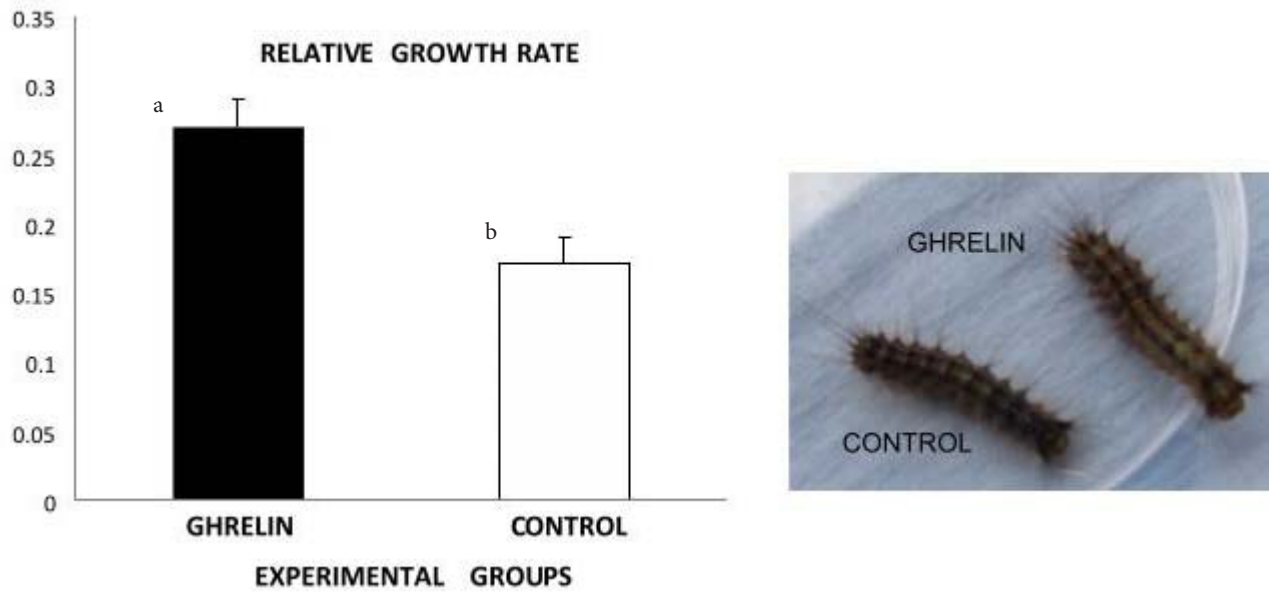

Fig. 1. Relative growth rate (RGR) after ghrelin treatment (black bar) / control experimental group (white bar) in 4th larval instar of *Lymantria dispar*; a and b show statistically different data between the groups (Fisher's LSD test).

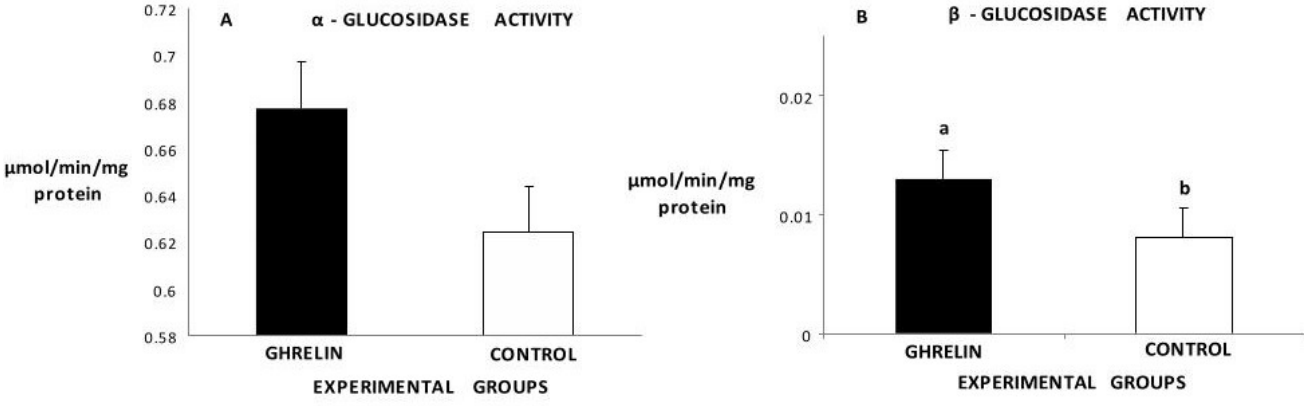

Fig. 2. A – the effects of ghrelin on α-glucosidase activity; B – the effects of ghrelin on β-glucosidase activity; a and b show statistically different data between the groups (Fisher's LSD test).

An increased release of digestive enzymes in many insects is related to feeding. For insects that feed continuously (including Lepidoptera larvae), enzyme activity is minimal at the time of molting and increases with the intensity of feeding (Lehane and Billingsley, 1996). Our results revealed elevation in the digestive enzyme activity in the midgut of 4th instar larvae of *Lymantria dispar* after ghrelin treatment (Fig. 2A, B), which was associated with an increased consumption rate. The efficiency of conversion of ingested food into biomass depends, among other factors, on the activity of digestive enzymes.

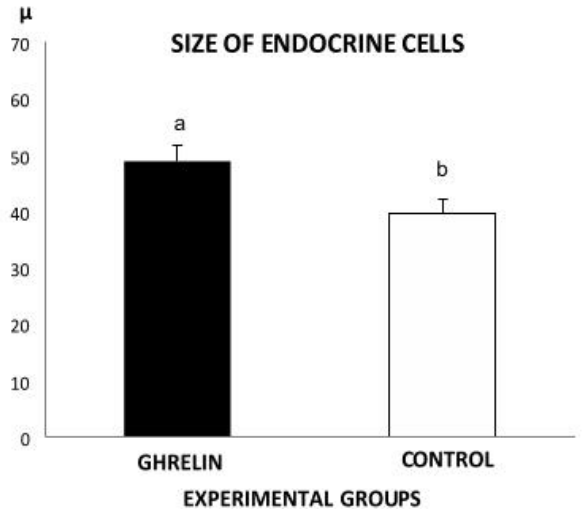

Fig. 3. The effect of ghrelin on the size of midgut endocrine cells; a and b show statistically different data between the groups (Fisher's LSD test).

Glycosidases are a type of digestive enzyme that have a critical role in the final stages of carbohydrate digestion: they hydrolyze α-D-(1,4)-glucose linkage such as p-nitrophenyl-α-D-glucoside in di- and oligosaccharide components. The present study clearly shows that the larvae of *Lymantria dispar* have higher α- and β-glucosidase activities in the midgut of ghrelin-treated than in the control group (Fig. 2). These enzymes are found in the alimentary canal of almost all polyphagous phytophagous insects and the type of food causes their selective effectiveness on the substrate. For example, Marinotti and James (1990) showed that α-glucosidase converts oligosaccharides into maltopentose in *Aedes aegypti* L. (Diptera: Culicidae). In honeybees, because of the presence of more sucrose than maltose, α-glucosidase hydrolyzes sucrose better than maltose, but in lepidopteran larvae, it is vice versa. β-glucosidase activity is linked with a large range of substrates and in some cases they are involved in insect resistance to plant defense chemicals (Terra et al., 1996). The effect of ghrelin on the *Lymantria dispar* midgut β-glucosidase activity is stronger than on the α-glucosidase. Hence, research into digestive enzymes, especially the regulation of enzyme activities, could be useful for understanding the roles of ghrelin in the physiology of nutrition in insects, as well as for controlling insect pests.

The insect midgut epithelium contains a significant proportion of endocrine cells. Their epitopes and ultrastructure are similar to mammalian gut endocrine cells, while quantitative changes in the levels of peptide hormones have been recorded in response to intensity of feeding (Endo and Nishiitsutsuji-Uwo, 1990; Zitnan et al., 1993). The cytological characteristics of endocrine cells in insect midgut express the nutritional status of ingested food and modulate digestive enzyme activity (Bede et al., 2007). The results of this research showed that the midgut endocrine cells were larger in ghrelin-treated caterpillars than in the control group, which indicate the intensification of their activity (Fig. 3).

The study of feeding mechanisms, digestion and growth, which is especially important in pests like *Lymantria dispar* L., could be a successful procedure in the development of a safe control strategy.

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