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Changes in Midgut and Brain Proteins in *M. funereus* Larvae Depending on Nutritive Substrate

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The response of *Morimus funereus* larvae to total starvation and refeeding with qualitatively different nutritive substrates (artificial diets supplemented with yeast as a source of B complex vitamins or with a digestibility reducer-tannic acid) was examined in this paper. Refeeding resulted in a compensatory increase of larval growth. Feeding and refeeding with qualitatively different nutritive substrates affected both quality and quantity of midgut and brain proteins. The observed differences suggest the possible switching of enzyme isoforms in *M. funereus* midgut and changes in synthesissecretion of neurohormones, depending on food presence and its nutritional value.

Key words: *Morimus funereus*, starvation, refeeding, midgut, brain proteins.

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The ability of an organism to survive, grow and reproduce hinges upon its capacity to obtain nutrients from the environment (SIMPSON & SIMPSON 1994). Prolonged action of an unbalanced diet can disturb the dynamic equilibrium of basic biological processes (OSORIO *et al.* 1997).

The use of artificial diets gives insight into the biological significance of particular classes of molecules during the life cycle. Xylophagous insects represent a suitable model system for the examination of the influence of nutritive substrates with inadequate or unbalanced profiles of primary nutritients. Wood mass is characterized by a high content of carbohydrates (starch, cellulose) and lignin while the percentage of nitrogen is quite low (MATTSON 1980). For *Morimus funereus* larvae sapwood represents an external medium and a nutritive substrate. Larvae reared on artificial diet have much shorter development (6.5 months) than larvae from natural conditions (3 years) (IVANOVIĆ *et al.* 1991). The activation of protocerebral neurosecretory neurons, intensified protein and lipid metabolism as well as an increase in proteolytic activity (NENADOVIĆ *et al*. 1989; IVANOVIĆ *et al.* 1992) could be the cause of this reduction.

Insect dietetics has become a very important research field. It comprises, besides nutrition in the more classical sense, also the effects of food on feeding behavior and the effects of specific compounds such as nutrients, antivitamins, digestibility reducers and toxins on survival, growth, development, and reproduction (REESE 1979).

The objective of the present paper was to investigate the response of larvae reared under constant laboratory conditions exposed to two physiological states: starvation and refeeding with different nutritive substrates. We examined the effect of artificial diets supplemented with yeast (as a source of B complex vitamins) or with a digestibility reducer (tannic acid) on larval mass and midgut and brain proteins in *M. funereus* larvae.

Material and Methods

Experimental groups and rearing conditions

M. funereus larvae were reared from hatching to molting into the 5th instar stage on an artificial diet

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(AD) (ROBERTS 1986) and under controlled laboratory conditions (constant temperature of 23C, relative humidity of 70% and in the dark). The control group of larvae was sacrificed on the 5th day of the 5th instar stage (ADc group). Larvae were exposed to a 7-day total starvation from the 5th to 12th day of the 5th instar stage – (TS group), and after that refed for 7 days (from 12th to 19th day of 5th instar stage) with the following substrates:

AD substrate – (RF group);

AD substrate with 1% yeast – (ADY group);

AD with 0.02% or 0.2% of tannic acid – (Ta or Tb group, respectively).

Each group consisted of 8 larvae. Relative changes in larval body mass were determined as a difference between their mass after the treatment and initial mass divided by initial mass ((Mt – Mo)/Mo).

Biochemical methods

After decapitation, midguts and brains were dissected on ice and weighed. Midguts and brains were homogenized in RIPA buffer, 1:5 wet wt/vol. (50 mM TRIS pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% Triton X-100, 0.1% SDS) and centrifuged at 20 000g for 5 min. SDS-PAGE electrophoresis was performed according to LAEMMLI (1970), on 13.5% and 16% gels. The gels were stained for proteins with 0.1% $AgNO_3$. Gels were destained in a 0.025% formaldehyde and 3% Na_2CO_3 solution. Sigma markers Mr 14.2-66 and 2.5-17 kD were used as standards.

Results

Changes in *M. funereus* larval body mass

The data on larval body mass changes in each experimental group are presented in Figure 1. Significance was estimated by Student's *t*-test for dependent samples. Statistical significance of the differences between experimental groups was evaluated by ANOVA and the LSD test. A significant change in body mass was noticed only for the TS group. Mass change in this group is significantly different from other experimental groups. Increase in larval body mass, although not significant, was detected after refeeding and addition of the yeast and both concentrations of tannic acid in AD.

Changes in midgut protein patterns

Differences in midgut protein patterns in the region of high Mr (14.2-66 kD) are shown in Figure 2A. The patterns from groups ADc, TS, RF and ADY are very similar. Addition of tannic acid (both concentrations) induced changes at several Mr. Two bands of about 66 kD were not detected only in groups Ta and Tb. Two new bands Mr of 55 kD appeared only in these two groups. A new protein band (50 kD) is present only in the Ta and Tb groups. The same situation was observed for protein band Mr of about 40 kD. In all experimental groups the most intensive protein bands were those Mr of about 45, 35 and 29 kD.

Changes in protein patterns with low-molecular mass, i.e., 17 - 2.5 kD (Fig. 2B) are obvious between the ADc, TS, RF and ADY groups on one

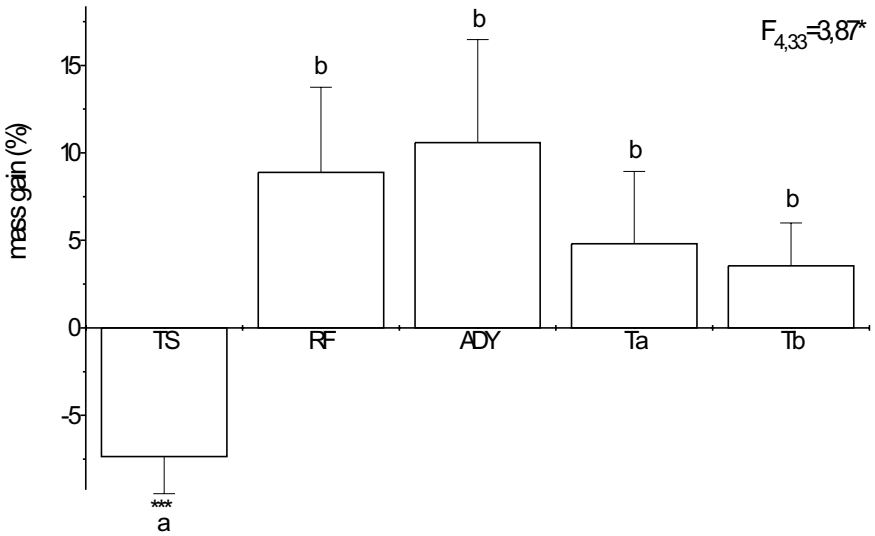

Fig. 1. Changes in body mass of *M. funereus* larvae after 7-day total starvation and 7-day refeeding. TS – changes in body mass after 7 day total starvation; RF – changes in body mass after 7 day refeeding with AD substrate; ADY– changes in body mass after 7 day refeeding with AD substrate and 1% yeast; Ta – changes in body mass after 7 day refeeding with AD substrate and 0.02% tannic acid; Tb – changes in body mass after 7 day refeeding with AD substrate and 0.2% tannic acid.

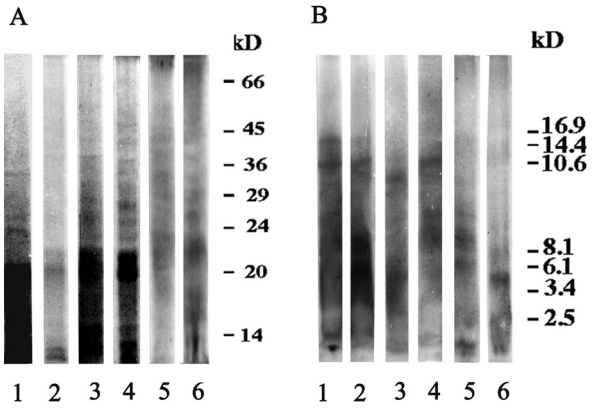

Fig. 2. Electrophoretic patterns of crude midgut homogenates of *Morimus funereus* larvae obtained by 13.5% (A) and 16% (B) SDS-PAGE. Lane 1 – control group, larvae sacrificed on 5th day, 5th instar; Lane 2-7 day total starvation; Lane 3-7 day refeeding with AD substrate; Lane 4-7 day refeeding with AD substrate and 1% yeast; Lane 5-7 day refeeding with AD substrate and 0.02% tannic acid; Lane 6-7 day refeeding with AD substrate and 0.2% tannic acid.

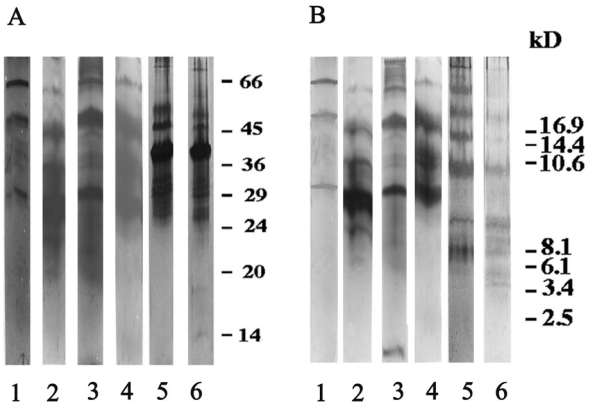

Fig. 3. Electrophoretic patterns of crude brain homogenates of *Morimus funereus* larvae obtained by 13.5% (A) and 16% (B) SDS-PAGE. Lane 1 – control group, larvae sacrificed on 5th day 5th instar; Lane 2-7 day total starvation; Lane 3-7 day refeeding with AD substrate; Lane 4-7 day refeeding with AD substrate and 1% yeast; Lane 5-7 day refeeding with AD substrate and 0.02% tannic acid; Lane 6-7 day refeeding with AD substrate and 0.2% tannic acid. Figure 3B presents the brain protein patterns in low-molecular masses region. In all groups only several bands were detected. After refeeding the Mr 15 kD band, present in the control group, disappeared. The same change occurred with band Mr of 12.5 kD. Both bands were detected in all other treatments. Refeeding also induced the disappearance of band Mr 10.6 kD. In the Ta and Tb groups the bands in the higher region are less intense compared to those in the lower Mr. In the Tb group bands of 2.51 to 8.1 kD are barely visible. Protein patterns of all other groups are very similar to each other.

side and the Ta and Tbs group on the other. Bands Mr of about 16.9 and a band of 10 kD detected in all groups were of slightly lower Mr in the Ta and Tb groups. These two groups were characterized by the presence of two bands Mr of 8 kD that could not be detected in other treatments. In the Ta group a completely new protein band Mr of 5 kD appeared.

Changes in brain protein patterns

The brain electrophoretic patterns are presented in Figure 3A (the region of high molecular masses, 16.5-2.51 kD)**.** In all experimental conditions pairs of bands were detected at 45, 36, 29, 24 and 20 kD. Differences in patterns between groups are negligible except for the absence of a band Mr 36 and 29 kD in the TS group. After refeeding the protein band of higher mass in that region was intensified in comparison to that of the lower, while in the control group the opposite is true. In all experimental groups the most intensive protein bands are in region between 24 and 20 kD.

Discussion

Developing larvae need uth2a constant food supply for growth, development and maintenance of homeostasis (REESE 1979). After a certain period of nutritional deficit, individuals can accelerate growth if conditions improve. The compensatory growth rates are usually regulated at optimal rather than maximal rates (METCALFE & MONAGHAN 2001). In *M. funereus* larvae, starvation and subsequent refeeding induce a compensatory reaction, which can be characterized by accelerated growth after total starvation (Fig.1).

Xylem fluid contains the lowest nitrogen concentration of any plant tissue (MATTSON 1980). Protein digestibility is also minimized. Insects' requirements in amino acids are qualitatively very similar (BRODBECK & STRONG 1987) but concentrations required for their growth and development exceed that found in plant tissues (MCNEILL & SOUTHWOOD 1978). A diet after Roberts, used in this experiment, contains 0.9% zein, considered a low-quality protein (KAROWE & MARTIN 1989). The results on mass changes obtained in this paper indicate that either *M. funereus* larvae are resistant to food deprivation or they have a short recovery time.

The presence of vitamins of the B complex in diet is important for riboflavin content, FAD and FMN, the coenzyme forms of vitamin B2. Protein and lipid synthesis, i.e. larval growth, depends on B vitamins (CHANG *at al*. 2000). The role of the vitamin B complex (present in yeast) in insect growth is shown in our experiment (Fig. 1).

Tannins are phenolic compounds which have an important role in protecting plant tissues from herbivore attack (FENNY 1976). They form insoluble

complexes with proteins, inhibiting enzyme activities and reducing digestibility of food proteins (GOLDSTEIN & SPENCER 1985). The larvae used in this experiment originated from imagoes collected in oak forest. A great amount of hydrolyzable tannins is present in sapwood of *Quercus sp*. (SCALBERT 1992). On the basis of changes in larval mass (Fig. 1), it may be presumed that tannic acid has a phagostimulatory effect on *M. funereus* larvae.

Protein bands Mr 20-40 kD were detected in the midgut of all experimental groups (Fig. 2A). This region is known as the Mr range of trypsin- and chymotrypsin-like forms of digestive enzymes in insects. The molecular masses for three trypsin-like enzymes in *M. funereus* larvae were determined to be 35, 46 and 54 kD (ĐURĐEVIĆ 1997). Protein bands of 35 and 45 kD are present in all groups. There may be enzyme forms present in *M funereus* midguts independent of environmental conditions. In *M. funereus* larvae crude midgut extracts and individual fractions obtained by gel filtration contained trypsin-like enzymes together with chymotrypsin and trypsin (VUJČIĆ *et al.* 1998). The 54 kD isoform, detected only in groups Ta and Tb (Fig. 2), may be a tannin resistant trypsin. XU and QIN (1994) have demonstrated different inhibition specificity of tannic acid toward proteases, which depends on the internal environmental conditions and affinity of digestive enzymes to free tannins from plant tissues. The differences in protein patterns (Fig. 2) suggest the possible switching of enzyme isoforms, depending on food presence and its nutritional value (like the presence of secondary metabolites, i.e. tannic acid).

IVANOVIĆ *et al.* (1998) have observed, that changes in proteolytic activity in *M. funereus* larvae, refed after 7-day starvation, strongly depend on the nutritive value of the diet. In larvae reared on Robert's diet, sensitivity to the switch in diet was lower at the level of proteolytic enzymes that remained at the control level, while amylolytic activity was elevated when larvae were refed with AD (IVANOVIĆ *et al.* 2002).

The protein bands in low Mr regions in *M. funereus* midgut protein patterns (Fig. 2B) could represent glicosilated proteins from a peritrophic matrix, the subunits of some enzymes, products of protein digestion or neuropeptides from midgut endocrine cells (EAST *et al.* 1993, NISHIITSUTSUJI-UWO *et al.* 1981; SEHNAL & ŽITNAN 1996).

A loss of protein bands from the region of insect allatotropin molecular masses (KATAOKA *et al.* 1989) was detected upon starvation in brain protein patterns (Fig. 3). These neurohormones are synthesized in medial neurosecretory cells and the *corpus cardiacum* is their neurohaemal site. Allatotropins stimulate the synthesis of juvenile hormones. Knowing that starvation induces a decrease in juvenile hormone synthesis (DE LA GARZA *et al.* 1991), this could point to changes of allotropic hormones during nutritional stress in *M. funereus* larvae. Other changes in brain protein patterns were detected in Mr regions of bursicon (about 40 kD) and prothoracicotropic hormones (24-30 kD and 5-10 kD) (ISHIZAKI & SUZUKI 1988). It is possible that different stress factors influence neurohormone synthesis and secretion in *M. funereus* larvae.

Feeding and refeeding with qualitatively different nutritive substrates obviously influenced both the quality and the quantity of midgut and brain proteins. These changes in midgut proteins may indicate that nutritional stress induces the switch in digestive enzyme isoforms (probably trypsin- and chymotrypsin- like enzymes). Changes in brain protein patterns suggest a role of the neuroendocrine system in response to environmental changes.

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