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Effects of Rearing Density on Larval Growth and Activity of Digestive Enzymes in *Lymantria dispar* L. (Lepidoptera: Lymantriidae)

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Density dependent responses of 4th, 5th and 6th instar gypsy moth larvae were studied at the level of larval mass, midgut loading and activities of three digestive enzymes (α-amylase, trypsin and leucine aminopeptidase). High density significantly reduced larval mass while midgut loading (expressed as relative midgut mass) did not change except in the 5th instar where it was increased at high density. Specific amylase and leucine aminopeptidase activities were not affected by crowding. Specific trypsin activity was on average higher in crowded than in isolated larvae. High density also affected the correlations between midgut protein content and activities of two proteolytic enzymes suggesting differences in regulatory mechanisms of insect digestion. The importance of these changes for survival under stressful conditions is discussed.

Key words: *Lymantria dispar*, density effects, fitness, α-amylase, trypsin, leucine aminopeptidase.

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Increased density is known to significantly affect fitness components, behaviour and metabolism in insects (PETERS & BARBOSA 1977; APPLEBAUM & HEIFETZ 1999). It may shorten (SIMMONDS & BLANEY 1986; CONNAT *et al*. 1991; TUCIĆ *et al*. 1991; TAMMARU *et al*. 2000) or prolong insect development (LORD 1998; ROBERTS 1998) while survival, body weight and fecundity are generally reduced at high density (WALL & BEGON 1986; TUCIC *et al.* 1991; KAZIMIROVA 1996; ROBINS & REID 1997; LORD 1998; ROBERTS 1998; HIRSCHBERGER 1999; HOOPER *et al*. 2003). Negative density effects may be attributed to starvation, accumulation of toxic waste products and/or mechanical interference (DYE 1984; ROBERTS 1998). Additionally, adaptive changes in response to crowding are possible in insects. These changes may be large and specific such as wing polyphenism (ZERA & RANKIN 1989) and melanization (SWORD 1999), or include weak responses whose adaptive nature is not immediately obvious (HAUKIOJA *et al*. 1988).

Density dependent responses at the level of hormones and neurohormones lead to increased respiration, mobilization of carbohydrates and lipids, and different expression of glycolytic and other enzymes (APPLEBAUM & HEIFETZ 1999). Higher lipid content (FERGUSON *et al*. 1997), induction of Hsp70 expression (SORENSEN & LOESCHCKE 2001), increased food consumption (SIMMONDS & BLANEY 1986; WEAVER & MCFARLANE 1990) and changes in host preference (SIMMONDS & BLANEY 1986) have also been described under crowded conditions.

The gypsy moth is a species with outbreaking population dynamics. Periodical eruptive increase in its populations leads to defoliation and diminished host leaf quality (VALENTIN *et al*. 1983; ROSSITER *et al*. 1988) which partly account for density dependant responses in gypsy moth performance and behavior. Gypsy moths from high density populations are characterized by changes in feeding rhythm (LANCE *et al*. 1986a) and coloration (PONOMAREV 1994), wider host range (BARBOSA 1978), higher mobility (BARBOSA *et al*. 1981), rapid development and smaller body size (LEONARD 1981). Physiological responses to crowding are poorly investigated in the gypsy moth. Furthermore, data on density effects on insect digestion physiology are almost lacking.

The present study was aimed to determine (1) density effects on larval mass as a fitness-related trait, and (2) density-dependant responses at the level of activities of three digestive enzymes (α-amylase, trypsin, leucine aminopeptidase) as a possible physiological basis of modified fitness; (3) the third objective was to reveal changes in the regulatory mechanisms of the digestive enzymes in relation to rearing density.

Material and Methods

Insects and rearing conditions

Egg masses used in this experiment were obtained from gypsy moths reared under laboratory conditions (23°C, *Quercus cerris* leaves, low density) for two generations and originated from an oak forest, at Despotovac (100 km south-east from Belgrade, Serbia).

Egg masses were kept at 4°C from December to April when they were set for hatching. Egg hairs were removed and eggs were surface sterilized for 5 min in 0.1% sodium hypochlorite, then rinsed in distilled water for 10 min and air dried. The hatched larvae were reared on oak leaves (*Quercus cerris* L.) at 23°C and 16:8 light:dark photoperiod. They were supplied with fresh leaves daily.

Larvae were reared in plastic cups (200 cm^3) at a density of 5 caterpillars per cup until molting into the 4^{th} instar. Then they were divided into two groups. The first group consisted of larvae kept under isolated conditions, i.e. one larva per cup, while in the second group larvae were kept under crowded conditions. A density of 5 larvae per cup was maintained during 4^{th}, 5^{th} and 6^{th} instar.

Preparation of crude midgut extracts

Larvae were weighed and killed 2 days after molting into the 4^{th} instar and 3 days after molting into the 5^{th} and 6^{th} instar. They were immobilized on ice and their midguts were removed by dissection in cold 0.9% NaCl solution. Midguts were weighed and relative midgut mass (RMM) was computed as the percentage of larval mass. According to JINDRA and SEHNAL (1989), RMM is a measure of the extent of gut filling.

The midguts were homogenized individually in a 10mM Tris-HCl buffer (pH 7.2, 1:10 wet wt/vol) for 30s. The homogenates were centrifuged at 10,000 rpm using a Sorvall centrifuge for 10min at 4°C. The supernatants (crude midgut extracts) were kept at -20°C until use.

Enzyme assays and protein determination

Amylase activity was determined by the dinitrosalicylic acid (DNSA) procedure (BERNFELD 1955) modified by DOANE (1969) at optimal temperature and pH previously described for *in vitro* activity of gypsy moth α-amylase (LAZAREVIĆ *et al*. 1998). The reaction was stopped by adding DNSA reagent and the color developed by heating in boiling water for 5 min. After dilution of samples with water, the release of reducing groups was evaluated by reading absorbance at 550 nm. Maltose was used as the standard. Trypsin and leucine aminopeptidase activity were determined using the chromogenic substrates BApNA (*N*-benzoyl--DL-arginine *p*-nitroanilide) and LpNA (L-leucine *p*-nitroanilide) at a final concentration of 1mM in 0.1M Tris-HCl (pH 8.0) at 25°C (ERLANGER *et al*. 1961; VALAITIS 1995). The release of *p*-nitroanilide was continually monitored at 405 nm using a Shimadzu UV-160 spectrophotometer. One enzyme unit corresponded to the hydrolysis of 1 μmol of substrate per minute.

Protein concentration was estimated according to LOWRY *et al*. (1951) using bovine serum albumin as the standard.

Statistical methods

Following the examination of normality and homogeneity of variance, appropriate ANOVA models were applied on arcsin-square root transformed values of RMM and the log transformed values of larval mass. To exclude body size effects on enzyme activity, log transformed data on digestive enzyme activities were analyzed by 2-way ANCOVA with the logarithm of larval mass as covariate, and density and larval instar as fixed factors (SOKAL & ROHLF 1981). Pearson's product-moment correlations were used for determining phenotypic correlations among larval mass, relative midgut mass, and specific activities of digestive enzymes as well as between midgut protein content and total activities of two proteolytic enzymes (expressed as U per ml of homogenate). Differences between correlation coefficients at low and high densities were tested by Z tests (ZAR 1984).

Results

Larval and midgut mass

High density significantly reduced larval mass. An increase in larval mass during development is less expressed in larvae reared under crowded conditions, although the interaction effect of rearing density and larval instar was marginally significant (Fig. 1).

Relative midgut mass (RMM) was not affected by high density (Fig. 1). The increase in RMM dur-

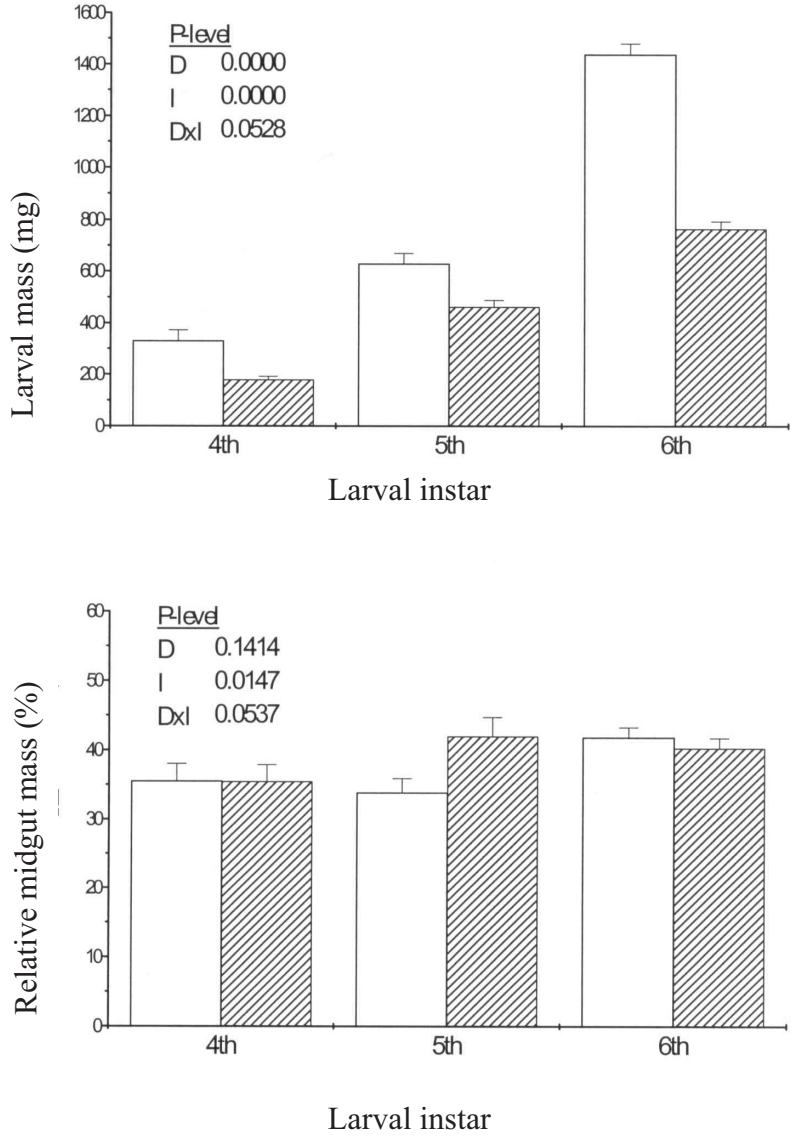

Fig. 1. Larval mass and relative midgut mass in 4th, 5th and 6th instar gypsy moths reared under low (hollow bars) and high density (striped bars). P-level represents the significance of main and interaction effects of rearing density (D) and larval instar (I) from two-way ANOVA. Bars represent means ±S.E.

Table 1

Two-way ANCOVA for the effect of density and larval instar on relative midgut mass (RMM) and specific activities of α-amylase (SAA), trypsin (STA) and leucine aminopeptidase (SLA) in the gypsy moth. Logarithm of larval mass is used as a covariate. Sum of squares are multiplied by 100

Source of variation	df	RMM			SAA			STA			SLA		
		SS	F	P-level	SS	F	P-level	SS	F	P-level	SS	F	P-level
Covariate	1	2.27	4.07	**0.0483**	19.14	24.33	**0.0000**	2.24	2.70	0.1062	5.79	4.84	**0.0321**
Density (D)	1	0.75	1.35	0.2506	0.75	0.95	0.3449	15.57	18.75	**0.0001**	0.58	0.49	0.4966
Instar (I)	2	0.69	0.62	0.5400	0.17	0.11	0.8993	1.15	0.70	0.5037	10.07	4.21	**0.0200**
D x I	2	3.59	3.22	**0.0472**	1.98	1.26	0.2929	16.29	9.80	**0.0002**	8.72	3.65	**0.0326**
Error	....	31.71	df=57		42.49	df=54		44.89	df=54		65.81	df=55	

Density and instar are fixed factors. Significant sources of variation are given in bold.

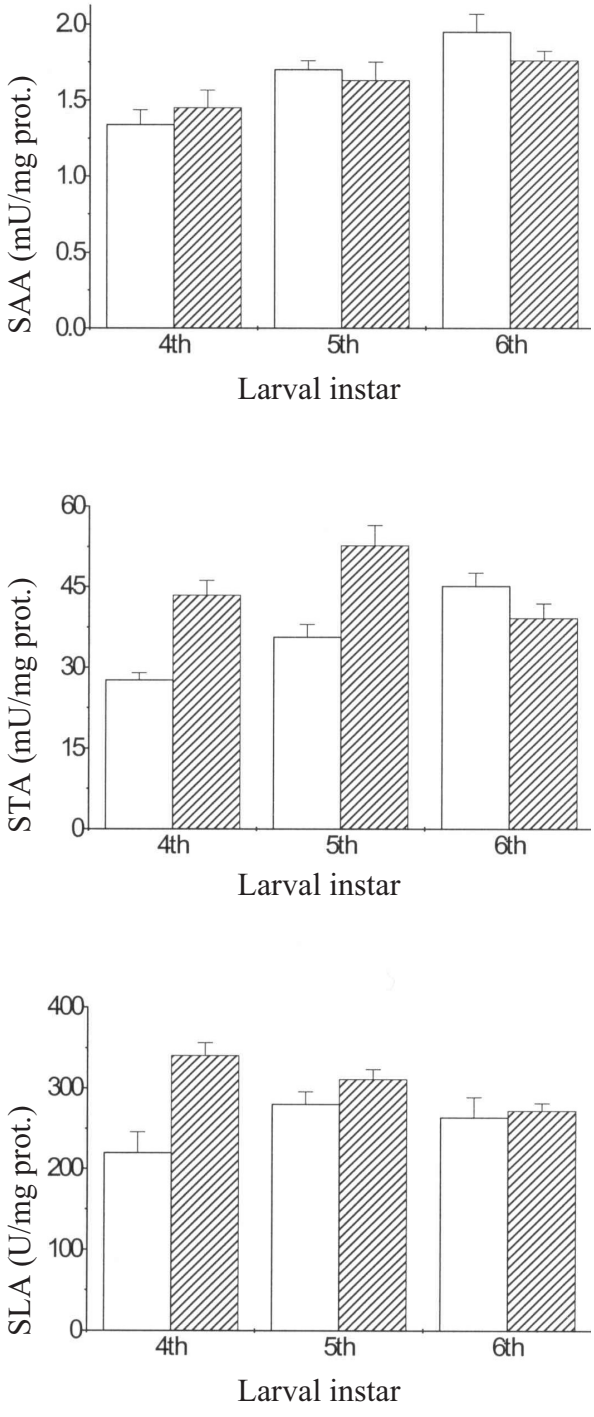

Fig. 2. Specific activities of α-amylase (SAA), trypsin (STA) and leucine aminopeptidase (SLA) in the midguts of 4th, 5th and 6th instar gypsy moth larvae reared under low (hollow bars) and high density (striped bars).

ing development could be attributed mainly to body size effects since two-way ANCOVA did not reveal a significant instar effect on RMM (Table 1). The density effect was not significant in 4th and 6th instar but 5th instar crowded larvae had larger midgut loading than isolated larvae (LSD test, $P<0.0063$).

Larger larvae reared at low density had larger gut loading. This correlation was lost at high density, although the difference between the environments was not significant (Table 2).

Digestive enzyme activities

The activity of α-amylase was not affected by high density or larval instar (Table 1). The trend of increased activity during development, which can be noticed in Figure 2, was the result of increased larval mass. The activity of α-amylase is correlated to larval mass both at low and high densities (Table 2).

Two-way ANCOVA revealed a significant density effect on trypsin activity (Table 1). The effect of density depended on larval instar (significant interaction term). The activity of trypsin was significantly higher in crowded 4th (LSD test, $P<0.0000$) and 5th instar larvae (LSD test, $P<0.0000$) than in isolated ones, while 6th instar larvae were not affected by rearing density (Fig. 2). A significant correlation coefficient between larval mass and trypsin activity was found in isolated larvae. This correlation was lost at high density (Table 2).

The activity of leucine aminopeptidase increased during the development of isolated larvae and decreased in crowded ones (Fig. 2). Significant instar and interaction terms were revealed by the two-way ANCOVA, while the density effect was not significant (Table 1). Only in the4th instar larvae was activity of leucine aminopeptidase higher at high than low density (LSD test, $P<0.0001$). At high density, larger larvae were characterized by lower activity of leucine aminopeptidase while there was no significant correlation LM-SLA at low density (Table 2).

Gut filling (RMM) was significantly correlated only with the activity of leucine aminopeptidase at low density. Significant positive correlations were found between SAA and STA both at low and high density as well as between STA and SLA at low density. Significantly positive correlations between midgut protein content and total activities of two proteolytic enzymes were found in crowded larvae but not in isolated ones. Differences between environments were significant (Table 2).

The relationship between activities of trypsin and leucine aminopeptidase was affected by density only in the 5th instar larvae. The leucine aminopeptidase to trypsin ratio was higher in isolated (8.029 ± 0.413) than in crowded larvae (6.126 ± 0.478), (LSD test, $P<0.0169$).

Table 2

Correlations (±S.E.) among larval mass (LM), relative midgut mass (RMM), specific activities of α-amylase (SAA), trypsin (STA) and leucine aminopeptidase (SLA), as well as between midgut protein content (MPC) and total activities of two proteolytic enzymes (trypsin- TA and leucine aminopeptidase- LA) in gypsy moths reared at low or high density. Differences between the environments were tested by Z-test

Correlations		Low density	High density	Z
LM	RMM	0.3912±0.1534 *	0.2255±0.1989	0.617
	SAA	0.6259± 0.1358 ***	0.4399±0.1833 *	0.680
	STA	0.6278±0.1355 ***	0.0555±0.2038	2.094*
	SLA	0.0295±0.1714	-0.4429±0.1830 *	1.739
RMM	SAA	0.2833±0.1669	0.1848±0.2006	0.360
	STA	0.1861±0.1710	-0.0152± 0.2041	0.736
	SLA	-0.3894±0.1580 *	-0.3309±0.1926	0.215
SAA	STA	0.7652±0.1156 ***	0.5402±0.1718 **	0.812
	SLA	0.2071±0.1729	-0.0673±0.2037	0.997
STA	SLA	0.4730±0.1534 **	0.2416±0.1981	0.938
MPC	TA	-0.1307±0.1726	0.4144±0.1858*	1.994*
	LA	0.2973±0.1650	0.8076±0.1204***	1.968*

* P>0.05, ** P<0.01, *** P<0.001

Discussion

Crowding induces morphological, physiological and behavioural responses in many insects. These phase transitions are triggered by aggregation pheromones and are hormonally regulated (APPLEBAUM & HEIFETZ 1999). Body size is usually decreased in response to high density which could be associated with decreased (TAMMARU *et al*. 2000) or increased development time with supernumerary molts (FESCEMYER & ERLANDSON 1993). Rapid development and smaller body size were shown in natural gypsy moth populations during outbreaks (LEONARD 1981) while laboratory investigations have shown an increase in the number of molts and longer development time in gypsy moths reared at high density (LEONARD 1968). Earlier studies also confirmed longer larval development time of crowded gypsy moths comparing to uncrowded ones (LAZAREVIĆ 2000). Results on larval mass in the present work (Fig. 1) are in agreement with the results of LEONARD (1968) and BELL *et al*. (1981). A moderately high density may shorten development time, but above a certain species-specific level, limited food and space lead to retarded development and decreased body size. As larvae in the experiment were fed ad libitum, decreased body size could be explained by allocation of resources towards energy metabolism.

Changes in food consumption rate and utilization efficiency underlie fitness modifications (KAUSE *et al*. 1999). The efficiency of conversion of ingested food into biomass depends, among other factors, on the activity of digestive enzymes. The food consumption rate is a behavioural trait but also encompasses physiological performance (FARRAR *et al*. 1989). In some insects, food consumption increases in response to high density (SIMMONDS & BLANEY 1986; WEAVER & MCFARLANE 1990). Laboratory tests with gypsy moths have not shown changes in feeding rhythms in response to high rearing density (LANCE *et al*. 1986b) but it is possible that, if resources are not limited, high density leads to an increase in food consumption and consequent increase in digestive enzyme activity. The rate of food consumption is correlated with the extent of midgut loading (JINDRA & SEHNAL 1989), i.e. relative midgut mass (RMM) determined in the present experiment. RMM was not affected by high rearing density except in the 5th instar larvae where it increased (Fig. 1).

The question arises if density-dependent changes in feeding behaviour could account for changes in enzyme activity. The results showed increased activity of the two proteolytic enzymes in some larval instars under grouped conditions. However, amylase activity was not affected by high density, which suggests the involvement of different regulatory mechanisms (Fig. 2; Table 1).

Synthesis and/or secretion of digestive enzymes could be controlled by neural, secretagogue or hormonal mechanisms (APPLEBAUM 1985).

Neural mechanisms regulate the immediate response of digestive enzymes to the mechanical stimulation of the midgut wall. It is expected that increased gut filling leads to increased enzyme secretion. However, none of the correlations between RMM and digestive enzyme activities was significant except the correlation between RMM and SLA at low density, which was significant and negative (Table 2).

The involvement of secretagogue mechanism is usually demonstrated by a significant positive correlation between a secreatagogue (a chemical from food) and enzyme activity (HOUSEMAN *et al*. 1985). It has been shown previously that protein secretagogue controls caseinolytic activity in isolated 5th instar gypsy moth larvae reared on suitable (oak) and unsuitable (locust tree) leaves (LAZAREVIĆ *et al*. 1994). The present work did not reveal secretagogue mechanism for trypsin and leucine aminopeptidase under low rearing density, although it became important under crowded conditions (Table 2). It is known that elastase is the dominant protease in the gypsy moth (VALAITIS 1995). However, the enzyme ratio could change under stressful conditions (LEMOS *et al*. 1992). It is possible that the ratio of elastase to other proteases is changed at high density. The change was shown for trypsin to leucine aminopeptidase ratio in the 5th instar gypsy moth larvae (see Results, last paragraph).

Hormonal mechanisms are involved in long term responses to a changing environment. Neurohormones from the *pars intercerebralis* affect activity of insect proteases and amylases directly or by changing food consumption (MURALEEDHARAN & PRABHU 1981; IVANOVIĆ *et al*. 1998; LEKOVIĆ *et al*. 2001). Their level is altered during development and under crowded conditions which may further regulate altered transcription and/or secretion of midgut proteases and amylases.

Positive correlations between amylase and trypsin activity obtained in the present study at both rearing densities (Table 2) can be explained by the fact that amylase and trypsin in Lepidoptera are parts of the same secretory vesicle, from which they are released together in response to various stimuli (SANTOS & TERRA 1984). The mechanisms of regulation of protease activity in the gypsy moth have not been investigated yet. Whether regulation occurs at the level of transcription or at the level of enzyme secretion and which hormones are involved in regulation of protease activity under grouped and isolated conditions are still unanswered questions.

Another important question to be answered is whether observed changes in digestive efficiency adequately match the changes in nutritional needs of gypsy moth larvae during development and under stressful conditions. The results have shown that the activity of the three digestive enzymes increased during development in larvae reared at low density (Fig. 2) which is in agreement with increased assimilation efficiency previously described by STOCKHOFF (1993). It is also well known that requirement for energy resources increases in older larvae (STOCKHOFF 1993) and during starvation (STOCKHOFF 1991).

Metabolic responses at the level of digestive enzyme activity could change the size of the assimilated pool of carbohydrates and amino acids in an adaptive manner, enabling higher survival during stress. Adaptive responses to crowding are increased food consumption, tolerance to toxic waste products (JOSHI 1997) and a higher allocation of resources towards carbohydrate rather than protein stores (STOCKHOFF 1991). Therefore, an increase in the activity of digestive enzymes involved in providing carbohydrates could be expected as an adaptive response to stress. Although the results did not show a significant density effect on amylase activity, a trend of increasing amylase activity during development remained in larvae reared at high density, while the developmental reaction norms for the two proteases were crossed (significant interaction term in 2-way ANCOVA, Table 1).

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