UC 577,1; 61 ISSN 0354-3447

Jugoslov. Med. Biohem. 22: 53–59, 2003 *Originalni naučni rad*
Original paper

NUTRITIVE STRESS EFFECTS ON GROWTH AND DIGESTIVE PHYSIOLOGY OF *LYMANTRIA DISPAR* LARVAE

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Summary: The effects of parental and offspring diet on larval growth, food consumption and utilization, and activities of three digestive enzymes (α-amylase, trypsin, leucine aminopeptidase) were examined in extremely polyphagous insect, the gypsy moth (*Lymantria dispar*). In parental generation, gypsy moth larvae were reared on oak (*Quercus cerris*) leaves as optimal host or beech (*Fagus silvatica*) leaves which contains flavonoids and alkaloids. In offspring generation, after molting into the 4th instar, they were either switched from oak to beech or remained on oak leaves. Decreased growth and food utilization efficiency, increased assimilation efficiency and activities of α-amylase and trypsin were recorded in larvae switched to beech leaves. Significant parental effects were demonstrated for fourth instar duration, weight of fifth instar larvae and specific activity of leucine aminopeptidase. Physiological, ecological and evolutionary context of the obtained results were stressed in the present paper.

Key words: Lymantria dispar, nutritive stress, maternal effect, nutritional indices, digestive enzymes

Introduction

The chemical composition of host plants significantly affects survival, development and reproduction of phytophagous insects (1). Food consumption and utilization link plant attributes with insect performance (2), and are frequently used as indirect measure of physiological resistance to nutritive stress (3).

Numerous studies in the field of nutritional physiology have reviewed effects of nutritive compounds (4, 5), and secondary metabolites (6) on insect responses. Some of the responses are adaptive such as preingestive increase in consumption of nutritionally poor food (7 9) or postingestive increase in activity of digestive enzymes (10 12). Efficient recognition and avoidance of food which contains toxic allelochemicals and induction of detoxification enzymes are examples of adaptive responses to toxicants (13 15).

Successful host plant use by phytophagous insects depends on their ability to adequately match spatial and temporal changes in chemical composition of host plants as well as changes in nutritional needs of insects during their development.

As a polyphagous phytophagous insect with outbreaking population dynamics, gypsy moth (*Lymantria dispar*) commonly encounters changes in food availability and quality. Behavioral and physiological plasticity enable survival under starvation and periodical exhaustion of suitable host plants. Food selection, compensatory feeding and adjustments in the efficiency of food utilization facilitate overcoming negative effects of imbalanced food. Experiments with artificial diets have demonstrated that the gypsy moth can self-select diet cubes according to its nutritional needs (16). Under field conditions it can benefit from switching between different host plant species (17 19), and possibly between conspecific host plants. Another form of phenotypic plasticity, important for survival in unpredictable environment, is nutritionally-based maternal effect which can be considered as mechanism of »transgenerational phenotypic plasticity« (20). This means that gypsy moth performance depends not only on its own nutritional environment but also the nutritional environment of the parental generation (21, 22).

Our experimental system included polyphagous insect, the gypsy moth, and two host plants, the oak

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(*Quercus cerris*) and the beech (*Fagus silvatica)* in variable parental/variable offspring diet situation. *Quercus species* are optimal hosts while gypsy moths fed on beech leaves have prolonged development time and lower pupal weight (23). This research was aimed to 1) examine the effects of switching to beech leaves on larval growth, food consumption and utilization indices, 2) to determine physiological responses at the level of activity of three digestive enzymes (α-amylase, trypsin and leucine aminopeptidase), and 3) to ascertain the presence of environmentally-based maternal effects on these physiological traits.

Materials and methods

Insects and rearing conditions

Gypsy moth egg masses used in this experiment were collected from oak trees in a mixed oak-beech forest Despotovac locality. Larvae hatched from these eggs (parental generation) were reared either on oak (*Quercus cerris*) or beech leaves (*Fagus silvatica*) at constant temperature (23 °C) and photoperiod (16:8 L:D). Larvae reared on beech leaves were exposed to strong selective pressure as most larvae (70%) died during the first instar. Next year, in the offspring generation, larvae were reared on oak leaves until molting into the fourth instar when they were either switch to beech leaves or remained on oak leaves. Four experimental groups were set depending on parental/offspring diet: OO-oak/oak; OB-oak/ beech; BO-beech/oak and BB-beech/beech.

Growth rates and nutritional indices

Eight to ten larvae were randomly assigned to four switching regimes, and placed individually in plastic cups (200 mL). Growth and nutritional indices were measured on the dry weight basis. Larvae were weighed at the beginning and end of the feeding trial, i.e., immediately after molting into the fourth instar and following voiding of the gut at the end of the instar. The duration of feeding trial was recorded (T_f) and frass and uneaten leaves were collected. Initial dry larval weight (W_0) was determined from a sample of 100 individuals, and final dry larval weight (W_f) was measured after drying at 65 °C for 48h. Similarly, relationship between wet and dry weight of the leaves was determined on leaf samples and amount of eaten leaves (W_i) was calculated as difference between initial dry weight and dry weight of uneaten leaves. Collected frass was also dried and weighed (W_f). The following formulae were used according to Waldbauer (24) and Farrar et al. (25) to calculate RGR (relative growth rate), RCR (relative consumption rate), AD (assimilation efficiency), ECI (efficiency of conversion of ingested food) and ECD (efficiency of conversion of digested food):

$$RGR=(W_t\ W_0)/(T_f*W_0)$$

$$RCR=W_i/(T_f*W_0)$$

$$AD=(W_i\ W_f)*100/W_i$$

$$ECI=(W_t\ W_0)*100/W_i$$

$$ECD=(W_t\ W_0)*100/(W_i\ W_f)$$

Activity of digestive enzymes

Eight to thirteen larvae within each experimental group were sacrificed on the third day of the fifth instar. Their midguts were dissected out in cold 154 mmol/L NaCl solution and homogenized individually in a 10 mmol/L Tris-HCl buffer (pH 7.2, 1:10 wet wt/vol) for 30 s. The homogenates were centrifuged at 10 000 rpm for 10min at 4 °C and supernatants, i.e., crude extracts were used for measuring enzyme activities.

α-amylase activity was determined by a modification of dinitrosalicilic acid procedure (26, 27) at pH and temperature optimal for gypsy moth amylase (28). Trypsin and leucine aminopeptidase activity were determined using the chromogenic substrates BApNA (*N*-benzoyl-DL-arginine *p*-nitroanilide) and LpNA (L-leucine *p*-nitroanilide), respectively (29). One unit of enzyme activity corresponds to the hydrolysis of 1 μmol of substrate per minute. Protein concentration was estimated according to Lowry et al. (30) using bovine serum albumin as a standard.

Statistical analysis

Nutritional indices and enzyme activities were analyzed by two way ANCOVA with parental and offspring diet (fixed effects) as main model terms. Following examination of homogeneity and normality of variance assumption ANCOVA models were applied on log transformed values. Larval weight was used as a covariate for the activity of digestive enzymes. In the analysis of nutritional indices numerator of the index was the dependent variable and denominator was the covariate (31).

Results

Larval growth and development

Switching to beech leaf diet significantly prolonged the duration of feeding period only in 4th instar gypsy moths whose parents ate oak leaves (Scheffe's multiple range test, $P<0.0018$). A two-way analysis of variance showed a significant interaction between parental and offspring diet *(Tables I, II)*.

Relative growth rate (RGR) was lower in 4th instar larvae switched to beech leaves *(Table I)*. Effect of switching on RGR was not dependent on nutritional experience of the parents which can bee seen from

Table I Means and standard errors for nutritional indices in 4th instar larvae of the gypsy moth depending on parental and offspring diet. N the number of larvae.

	OO	OB	BO	BB
N	10	8	9	10
Tf (days)	3.10 ± 0.18	4.38 ± 0.18	3.44 ± 0.18	3.80 ± 0.25
RGR (mg/mg/day)	0.78 ± 0.05	0.36 ± 0.02	0.81 ± 0.04	0.41 ± 0.03
RCR (mg/mg/day)	5.46 ± 0.43	5.78 ± 0.35	6.31 ± 0.41	6.16 ± 0.55
AD (%)	34.06 ± 1.16	39.94 ± 3.09	31.15 ± 1.36	35.94 ± 2.92
ECI (%)	14.55 ± 0.66	6.30 ± 0.35	13.04 ± 0.60	6.78 ± 0.37
ECD (%)	42.84 ± 1.66	16.74 ± 2.03	42.73 ± 3.06	19.81 ± 1.60

Table II Mean squares (×100) from two-way ANCOVA for nutritional indices where logarithms of initial weight (a), consumption (b) and consumption minus frass, i.e., assimilation (c) are used as covariates. Mean squares (×100) from two-way ANOVA are presented for T_f. Significant effects are highlighted using bold.

Source of variation	df	T_f		RGR[a]		RCR[a]		AD[b]		ECI[b]		ECD[c]	
		MS	P	MS	P	MS	P	MS	P	MS	P	MS	P
Covariate	1			2.55	**0.0000**	2.08	**0.0000**	6.43	**0.0000**	5.47	**0.0000**	3.63	**0.0000**
Parental diet	1	0.05	0.7071	0.14	0.1569	0.23	0.1210	0.21	0.0912	0.003	0.7980	0.16	0.2279
Offspring diet	1	8.20	**0.0005**	5.25	**0.0000**	0.04	0.5046	0.33	**0.0342**	9.71	**0.0000**	15.69	**0.0000**
Interaction	1	3.04	**0.0265**	0.03	0.4975	0.04	0.5112	0.001	0.9072	0.12	0.1231	0.04	0.5500
Error	32	0.56		0.07		0.09		0.07		0.05		0.11	

Table III Means and standard errors for larval weight (W_L), and specific activities of α-amylase (SAA), trypsin (STA) and leucine aminopeptidase (SLA) in 5th instar larvae of the gypsy moth depending on parental and offspring diet. N- the number of larvae.

	OO	OB	BO	BB
N	13	8	8	10
W_L (mg)	627.54 ± 42.08	442.88 ± 22.70	752.56 ± 102.77	669.70 ± 84.04
SAA (U/mg prot.)	1.71 ± 0.06	1.95 ± 0.09	1.36 ± 0.08	1.96 ± 0.06
STA (mU/mg prot.)	35.64 ± 2.39	67.17 ± 3.72	32.53 ± 2.35	68.32 ± 4.99
SLA (mU/mg prot.)	279.97 ± 15.88	285.48 ± 21.55	222.09 ± 14.32	245.22 ± 16.17

Table IV Mean squares (× 100) from two-way ANCOVA for specific activities of α-amylase (SAA), trypsin (STA) and leucine aminopeptidase (SLA) where the logarithm of larval weight is used as a covariate. Mean squares (× 100) from two-way ANOVA are presented for fresh larval weight (W_L). Significant effects are highlighted using bold.

Source of variation		W_L		SAA		STA		SLA	
	df	MS	P	MS	P	MS	P	MS	P
Covariate	1			0.39	0.2800	0.004	0.9502	1.61	0.1535
Parental diet	1	1.26	**0.0117**	1.13	0.0705	0.26	0.6100	7.71	**0.0030**
Offspring diet	1	1.10	**0.0181**	7.65	**0.0000**	68.78	**0.0000**	1.45	0.1747
Interaction	1	0.21	0.2862	2.73	**0.0064**	0.25	0.6151	0.07	0.7617
Error	34	0.18		0.32		0.98		0.75	

non-significant interaction term in two-way ANCOVA *(Table II)*.

Both parental and offspring diet significantly influenced larval wet weight measured on the 3rd day of the 5th instar. Larvae were larger when parents were fed with beech leaves, and a decrease in larval weight in response to switching was not significant in this group *(Tables III, IV)*.

Food consumption and utilization

Neither parental nor offspring diet affected relative consumption rate in 4th instar gypsy moths when consumption was calculated relative to the initial larval weight *(Tables I, II)*. If average larval weight over the duration of feeding period ($W_g=\sqrt{W_0*W_f}$) was used, ANOVA on log transformed values of RCR revealed a significant effect of offspring diet ($F=8.56$, $P<0.0062$). In this case, consumption was increased in response to switching to beech leaves (12). However, according to Farrar et al. (25) such measure of consumption rate encompasses not only behavior but also growth which depends on assimilation efficiency. Assimilation efficiency was significantly increased in larvae switched to beech leaves, while efficiency of conversion of ingested and digested food were significantly lower in this group *(Tables I, II)*. On the whole, parental diet had no effect on nutritional indices, and offspring diet affected all indices except relative consumption rate.

Amylase, trypsin and leucine aminopeptidase

Specific activities of α-amylase and trypsin were significantly increased in 5th instar larvae switched to beech leaves *(Table III)*. The lowest activity of α-amylase was recorded in BO group which was significantly different from OO (Scheffe's test, $P<0.0053$), OB ($P<0.0001$) and BB group ($P<0.0000$). The effect of parental diet on amylase activity was marginally significant. The plasticity of response to beech leaves was greater when parents were fed with beech leaves which was revealed by significant interaction term in two-way ANCOVA *(Table IV)*. Specific activity of trypsin was not affected by parental diet while activity of leucine aminopeptidase was significantly lower in larvae whose parents ate beech leaves *(Tables III, IV)*.

Discussion

Host plant effects on gypsy moth performance and its extremely polyphagous feeding habit have been well described (17, 19, 23, 32, 33). Using combine results of various tests (average defoliation, larval growth and survival, larval foliage preference etc.) Liebhold et al. (34) have ranked *Fagus silvatica* as intermediary suitable host plant. Beech leaves contain flavonoids and alkaloids (35), the synthesis of which begins a week after bud break contributing to strong antixenotic and antibiotic effects of beech leaves on 1st instar larvae (36). Young larvae are sensitive to *Fagus silvatica,* but older larvae can successfully metabolize beech leaves, and beech forests can be defoliated during outbreaks (37, 38). Decreased survival and pupal weight, and increased development time have been shown in gypsy moths reared on beech leaves through entire development (23, 39) or switched to beech leaves in older instars (12). Our results confirmed negative effects of beech leaves on larval growth and development *(Tables I, III)*.

We showed that relative growth rate (RGR) was lower in 4th instar larvae switched to beech leaves *(Table I)*. Analysis of nutritional indices helps us understand behavioral and physiological basis of such response. Mathematically and biologically RGR is the product of RCR and ECI which further depends on AD and ECD. Decreased growth could be a consequence of either decreased consumption (RCR) or utilization (ECI) or both. Another cause may be increased instar duration when increased amount of ingested food must be allocated to maintenance metabolism. As can be seen from *Tables I* and *II* switching to beech leaves did not change RCR while the duration of feeding period was prolonged only in larvae whose parents ate oak leaves. Considering unchanged RCR and T_f, concomitant decrease in RGR and ECD could be explained by a higher metabolic cost of processing of food which contains allelochemicals. Processing costs are associated with induction mechanisms at the level of digestion and detoxification.

Induction of superoxide dismutase, glutathione-S-transferase and microsomal polysubstrate monooxigenase is mechanism of defense against flavonoids and alkaloids (40, 41). Additionally, numerous papers have reported that activity of digestive enzymes responds to food composition and volume (10, 42 50). Induction of amylase and trypsin was shown in 5th instar gypsy moths switched to beech leaves *(Table III)*.

In 4th instar larvae, increased AD in response to beech leaves *(Table I)* could also be a result of changes at the levels of digestive enzymes. Apparently, the increase in AD could not compensate for the decrease in ECD which consequently led to reduced growth rate. Growth reduction is general response of phytophagous insects to switching to a new host plant (32, 51, 52).

Parental effects were demonstrated for the duration of feeding period (T_f), weight of fifth instar larvae (W_L) and specific activity of leucine aminopeptidase (SLA) while parental effects on assimilation efficiency (AD) and specific amylase activity (SAA) were marginally significant ($P<0.1$) *(Tables II, IV)*. Parental nutrition influenced sensitivity of T_f to beech leaves *(Table II)*. Significant interaction between parental and offspring diet has also been obtained for larval development time in the gypsy moth (21). The question arises what physiological mechanisms account for these changes. It is known that instar duration depends on relationship between juvenile hormone and ecdysone. Genes which determine this relationship could be subjected to selection (53). It is possible that strong selection during parental generation favourized individuals with shorter 4th instar.

Results on the weight of 5th instar larvae showed the advantage of large body size in stressful environment *(Tables III, IV)*. The weight of larvae was greater if their parents ate beech leaves. Although beech leaf diet in parental generation provoked a decrease in

body size (12), through negative maternal effect these smaller individuals may produce larger offspring. Larger body size is associated with higher fitness, i.e., higher fecundity, flying and mating ability, stress tolerance, etc. (54 56).

In conclusion, plasticity of physiological responses enables adjustment to variable nutritional environment in one generation while nutritionally-based maternal effects as »transgenerational phenotypic plasticity« enable time delay in responses of insect population (22). Via changing quality of eggs, parental nutrition may affect population dynamics (57) and trait evolution (58). Researches on plastic responses to nutritive stress are important for predicting insect outbreaks and understanding mechanisms of host plant specialization.

Acknowledgements. This work was supported by Ministry of Science, Technology and Development of Serbia, grant No. 1615.

EFEKAT NUTRITIVNOG STRESA NA RAST I FIZIOLOGIJU VARENJA KOD LARVI *LYMANTRIA DISPAR*

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Kratak sadržaj: Kod gubara *(Lymantria dispar)*, izrazito polifagnog insekta, ispitivan je efekat ishrane u roditeljskoj i potomačkoj generaciji na rast larvi, konzumaciju i utilizaciju hrane i aktivnost tri digestivna enzima (α-amilaze, tripsina, leucin aminopeptidaze). Larve gubara su u roditeljskoj generaciji gajene na lišću hrasta *(Quercus cerris)* kao optimalnom domaćinu ili lišću bukve *(Fagus silvatica)* koje sadrži flavonoide i alkaloide. U potomačkoj generaciji, posle ulaska u IV stupanj, larve su ili pebačene sa hrastovog na bukovo lišće ili su nastavile da se hrane hrastovim lišćem. Kod larvi prebačenih na ishranu bukovim lišćem pokazan je smanjen rast i efikasnost utilizacije hrane, povećana efikasnost asimilacije i aktivnost α-amilaze i tripsina. Značajan parentalni efekat je dobijen za trajanje IV stupnja, težinu larvi u V stupnju i specifičnu aktivnost leucin aminopeptidaze. U ovom radu je istaknut fiziološki, ekološki i evolucioni kontekst dobijenih rezultata.

Ključne reči: Lymantria dispar, nutritivni stres, materinski efekat, indeksi ishrane, digestivni enzimi.

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Received: January 10, 2003

Accepted: February 15, 2003