EFFECTS OF TANNIC ACID ON TRYPSIN AND LEUCINE AMINOPEPTIDASE ACTIVITIES IN GYPSY MOTH LARVAL MIDGUT

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Abstract – The effects of allelochemical stress on genetic variations in the specific activities of gypsy moth digestive enzymes (trypsin and leucine aminopeptidase) and relative midgut mass (indirect measure of food consumption), as well as variability in their plasticity, were investigated in fifth instar gypsy moths originating from two populations with different trophic adaptations (oak and locust-tree forests). Thirty-two full-sib families from the *Quercus* population and twenty-six full-sib families from the *Robinia* population were reared on an artificial diet with or without supplementation with tannic acid. Between population differences were observed as higher average specific activity of trypsin and relative midgut mass in larvae from the *Robinia* population. Significant broad-sense heritabilities were observed for the specific activity of trypsin in the control state, and for specific activity of leucine aminopeptidase in a stressful environment. Significantly lower heritability for relative midgut mass was recorded in larvae from the *Robinia* population reared under stressful conditions. Significant variability of trypsin plasticity in larvae from both populations and significant variability of leucine aminopeptidase plasticity in larvae from the *Robinia* population point to the potential for the evolution of enzyme adaptive plastic responses to the presence of stressor. Non-significant across-environment genetic correlations do not represent a constraint for the evolution of enzyme plasticity.

Key words: Gypsy moth, tannic acid, digestive enzymes, phenotypic plasticity

INTRODUCTION

The complex and variable chemical composition of host plants is an aspect of plant defense and represents a challenge for insect feeding. Phytophagous insects have evolved counter-adaptations to the defensive mechanisms of their host plants (Mello and Silva Filho, 2002). The successful use of host plants and coping with various host-specific allelochemicals depend on insect behavioral and physiological plasticity. As a component of a plant's "quantitative" defense, tannins play an important role in defense against phytophagous insects by deterrence and/or

toxicity. Tannin structural heterogeneity causes variations in their biological activity, which also depends on insect gut pH, surfactants and permeability of the peritrophic membrane. In insects with high gut pH (e.g. larvae of Lepidoptera), tannins are prone to oxidize and form semiquinone radicals, quinones and other reactive oxygen species. Their toxicity results from the production of high ROS levels (Barbehenn and Constabel, 2011). The gypsy moth, one of the most serious insect pests in forests of the northern hemisphere, is denoted as a tannin-tolerant herbivore (Barbehenn et al., 2009). However, higher levels of tannins in leaves or its high concentration in an

artificial diet have been shown to influence gypsy moth fitness (Rossiter, 1988; Bourchier and Nealis, 1993). We previously reported the adverse effects of a tannin-supplemented rearing diet on the life history traits and specific activity of some digestive enzymes in gypsy moth larvae originating from oak forest as a suitable, and locust-tree forest as a marginally suitable habitat (Mrdaković et al., 2011, 2013). Both oak and locust-tree leaves contain various allelochemicals (Barbosa and Krischik, 1987), including tannins (Montgomery, 1986; Rakesh et al., 2000). However, the tannin content is higher in the latter (Unruh-Snyder et al., 2007). More than 50 years of gypsy moth evolution on oak and locust-tree host plants (Sidor and Jodal, 1983) has led to significant divergence among these populations with regard to life history, behavioral and physiological traits (Perić Mataruga et al., 1997; Lazarević et al., 2002, 2003). Recently, we reported on differences among oak and locust-tree populations of gypsy moth larvae with regard to the activities of total protease, a-glucosidase and lipase, and significant variabilities in their plasticity in response to dietary tannic acid (Mrdaković et al., 2013). In this work, we examined among-population differences in the response of two gypsy moth digestive proteases, trypsin and leucine aminopeptidase, to allelochemical stress. We estimated the genetic variation and variation of phenotypic plasticity in the enzyme responses to dietary tannic acid, and assessed possible constraints on the evolution of optimal plastic responses. As an increase in consumption represents an adaptive pre-ingestive mechanism to cope with nutritionally inadequate food, quantitative genetic analysis was applied on the relative midgut mass of gypsy moth larvae, as an indirect measure of larval consumption.

MATERIALS AND METHODS

Insect rearing

The gypsy moth *Lymantria dispar* L. is physiologically suited for adult aphagy, and females emerge with adequate energy reserves to develop and lay most of their potential eggs. Since in low-density populations females rarely remate, a single egg mass is the product of a single mating and represents a full-sib family. As we previously described (Mrdaković et al., 2011), gypsy moth larvae were hatched from thirty-two egg masses collected from an oak forest, referred to as the *Quercus* population (locality "Bogovadja", 70 km southwest of Belgrade), and from twenty-six egg masses collected from a locust-tree (*Robinia pseudoacacia* L.) forest, referred to as the *Robinia* population (locality "Bagremara", 122km northwest of Belgrade). From both populations, within each egg mass 7-9 larvae (unbalanced full-sib design) were randomly assigned to a group fed on the control diet, a high wheat-germ-based (HWG) diet of the gypsy moth (O'Dell et al., 1985), and a group fed on the HWG diet containing 5% tannic acid (Tannic Acid (lot number 32K1248), Sigma-Aldrich). Four experimental groups were established: *Quercus* larvae fed on the control diet (QC), *Quercus* larvae fed on the tannin-supplemented diet (QT), *Robinia* larvae fed on the control diet (RC), and *Robinia* larvae fed on the tannin-supplemented diet (RT).

Enzyme assays and relative midgut mass measurement

Larvae were weighed and killed 3 days after molting into the fifth instar. Larval midguts were removed, weighed and kept in a freezer at -20°C until homogenization. Relative midgut mass (RMM) was calculated as the percentage of larval mass that, according to Jindra and Sehnal (1989), represents a measure of the extent of the gut fulfillment, i.e. indirect measure of food consumption. The midguts were homogenized individually on ice in 0.15 M NaCl (final tissue concentration was 100 mg/ml) for 30 s, and then centrifuged at 10,000 g, for 20 min at 4°C. The supernatants (crude midgut extracts) were used for determination of the specific activities of trypsin (TRY) and leucine aminopeptidase (LAP) (Erlanger et al., 1961). Specific enzyme activities were expressed as U/mg of midgut protein. One unit of enzyme activity corresponds to the hydrolysis of 1μmol of substrate per minute. Protein concentrations were determined according to Bradford (1976), using bovine serum albumin as the standard.

Statistical methods

Following the examination of homogeneity and normality of variance, appropriate ANOVA models were applied on arcsin-square root transformed values of RMM and the log-transformed values of enzyme activities. Enzyme activities and relative midgut mass were analyzed by two-way ANOVA with population origin and tannin treatment as the fixed factors. The significance of "population × treatment" interaction tests for the presence of population-level specialization to tannins. Broad-sense heritability was calculated by one-way ANOVA, using the formula for unbalanced full-sib design: $H^2=2V_g/(V_g+V_{error})$, (Becker, 1984). Comparison of heritabilities between the diets within each of the populations (QC-QT; RC-RT), and between two populations on each diet (QC-RC; QT-RT), were performed by z-test. Two-way ANOVA with family and treatment as factors was carried out in both populations. Treatment was fixed and family was a random factor. Significant "family × treatment" interaction revealed within-population genetic variation for plasticity in response to tannic acid present in a diet. By norms-of-reaction plots we depicted the differences in plastic responses of 32 full-sibs from the *Quercus* and 26 full-sibs from the *Robinia* populations. Genetic correlations across different environments (control and diet with 5% tannic acid) were calculated as Pearson's product-moment correlations, using family means of the traits (Sokal and Rohlf, 1981). All estimates came from the full-sib design and contained contributions from nonadditive genetic variance, maternal and environmental effects.

RESULTS

The specific activity of trypsin (TRY) was shown to be sensitive to dietary tannic acid, while leucine aminopeptidase showed a tendency to increase activity in response to dietary tannin in both analyzed populations. The relative midgut mass (RMM) of gypsy moth larvae did not change significantly in the presence of the stressor, neither in the *Quercus* nor in the *Robinia* population. However, the highest value of RMM was recorded in larvae from the *Robinia* population reared on the control diet, while on the tannin-supplemented diet a higher RMM was recorded in *Robinia* than in *Quercus* larvae (1-way ANOVA, $F_{3,782}=9.974$, P=0.0000) (Table 1). Two-way ANOVA with population and tannin treatment as the factors, revealed significant population and significant treatment effects on the specific activity of trypsin (TRY) and relative midgut mass (RMM) of larvae. A significant treatment effect was recorded for the specific activity of leucine aminopeptidase (LAP). Differences in the response of local populations to tannin treatment were not obtained for the analyzed traits (non-significant "Population × Treatment" interaction term) (Table 2).

Broad-sense heritability (Table 3A) for the specific activity of trypsin (TRY) was significant in both *Quercus* and *Robinia* larvae reared on the control diet, while the specific activity of leucine aminopeptidase (LAP) was significant in all groups except *Quercus* larvae reared on the control diet. Broad-sense heritability for relative midgut mass (RMM) was not significant only in *Robinia* larvae reared on the tannin-supplemented diet. Comparison of heritabilities (Table 3B) for examined traits between the diets within each of the populations (QC-QT; RC-RT), and between two populations on each diet (QC-RC; QT-RT), revealed significantly higher heritability of RMM in *Robinia* larvae reared on the control than in those reared on the tannin-supplemented diet.

Significant family and treatment effects, as well as significant interaction, were recorded for the specific activity of trypsin (TRY) in both *Quercus* and *Robinia* populations (Table 4). Twenty-six families from the *Quercus* and 16 families from the *Robinia* populations displayed reduced specific activity of TRY in larvae reared on the tannin-supplemented diet (Fig. 1A). Significant family effects were recorded for the specific activity of leucine aminopeptidase (LAP) in both *Quercus* and *Robinia* populations, while a significant interaction effect was recorded for LAP activity in *Robinia* larvae (Table 4). Significant interaction term points to significant among-family differences in the sensitivity of the enzyme activities to the presence of tannic

Table 1. Mean values and standard errors of specific activity of trypsin (TRY) and leucine aminopeptidase (LAP), and of relative midgut mass (RMM) of gypsy moth larvae from *Quercus* and *Robinia* populations, reared on control (C) and on tannin-supplemented (T) diets. Values marked with different letters differ significantly (Sheffe's multiple range test).

| | *Quercus* population | | | | | | *Robinia* population | | | | | |
| | C | | | T | | | C | | | T | | |
	N	X ±	SE	N	X ±	SE	N	X ±	SE	N	X ±	SE
[1]TRY	213	0.387	0.010[ab]	239	0.307	0.007[c]	180	0.418	0.013[a]	198	0.353	0.009[b]
[1]LAP	218	0.633	0.014[a]	244	0.671	0.015[a]	179	0.622	0.014[a]	189	0.656	0.014[a]
RMM(%)	195	22.448	0.314[ac]	229	21.371	0.323[a]	175	24.049	0.420[b]	187	22.883	0.363[bc]

([1]Results for TRY and LAP- Mrdaković 2010, and previously submitted 2013)

Table 2. Mean squares (x10³) from two-way ANOVA (with population origin and treatment as fixed factors) for specific activity of trypsin (TRY), and leucine aminopeptidase (LAP), and for relative midgut mass (RMM) of gypsy moth larvae from *Quercus* and *Robinia* populations, reared on control and on tannin-supplemented diets; df – degree of freedom; *P<0.05, **P<0.01, ***P<0.001.

| | TRY | | | LAP | | | RMM | | |
Source of variation	df	MS (x10³)	F	df	MS (x10³)	F	df	MS (x10³)	F
Population	1	475.432	16.784***	1	5.382	0.285	1	0.495	17.609***
Treatment	1	1269.148	44.804***	1	107.348	5.694*	1	0.274	9.768**
Interaction	1	53.769	1.989	1	0.334	0.018	1	0.003	0.010
Error	826	28.327		826	18.853		782	0.028	

Table 3. A. Broad sense heritability (H²±SE) of specific activity of trypsin (TRY) and leucine aminopeptidase (LAP), and of relative midgut mass (RMM) in gypsy moth larvae from two populations reared on control (C), and on tannin-supplemented (T) diets. Significance of heritability was determined using t-test; **B.** t-values obtained by comparison of trait heritabilities between the diets within each of the populations (QC-QT; RC-RT), and between the two populations on each diet (QC-RC; QT-RT), performed on z-transformed values of heritabilities.
*P<0.05, **P<0.01.

| | *Quercus* population | | | | *Robinia* population | | | | **B.** Comparison of H² | | | |
| A. | C | | T | | C | | T | | | | | |
	H² ±	SE	H² ±	SE	H² ±	SE	H² ±	SE	QC-QT	RC-RT	QC-RC	QT-RT
TRY	0.472	0.147**	0.230	0.113	0.306	0.142*	0.133	0.107	1.223	0.817	0.786	0.499
LAP	0.215	0.117	0.272	0.117*	0.430	0.158*	0.351	0.145*	0.414	0.350	1.131	0.372
RMM	0.413	0.150**	0.482	0.146**	0.614	0.177**	0.164	0.119	0.356	**2.120***	1.048	1.526

Table 4. Mean squares (x10³) from two-way ANOVA (with treatment as fixed and family as the random factor) for specific activity of trypsin (TRY) and leucine aminopeptidase (LAP), and for relative midgut mass (RMM), of gypsy moth larvae from *Quercus* and *Robinia* populations, reared on control and on tannin-supplemented diets; df – degree of freedom; *P<0.05, **P<0.01, ***P<0.001.

| | Source of variation | TRY | | | LAP | | | RMM | | |
		df	MS (x10³)	F	df	MS (x10³)	F	df	MS (x10³)	F
Quercus population	Family	31	72.323	3.019***	31	42.496	2.411***	31	0.058	2.873***
	Treatment	1	989.074	18.030***	1	81.047	2.873	1	0.234	4.020
	Interaction	31	54.856	2.290***	31	28.214	1.601	31	0.058	2.891***
	Error	388	23.956		398	21.681		360	0.020	
Robinia population	Family	25	57.195	2.352***	25	51.241	3.644***	25	0.102	4.158***
	Treatment	1	372.277	8.900**	1	70.221	2.500	1	0.098	2.740
	Interaction	25	41.830	1.720*	25	28.084	1.977**	25	0.036	1.447
	Error	326	24.320		316	14.062		310	0.025	

A.

B.
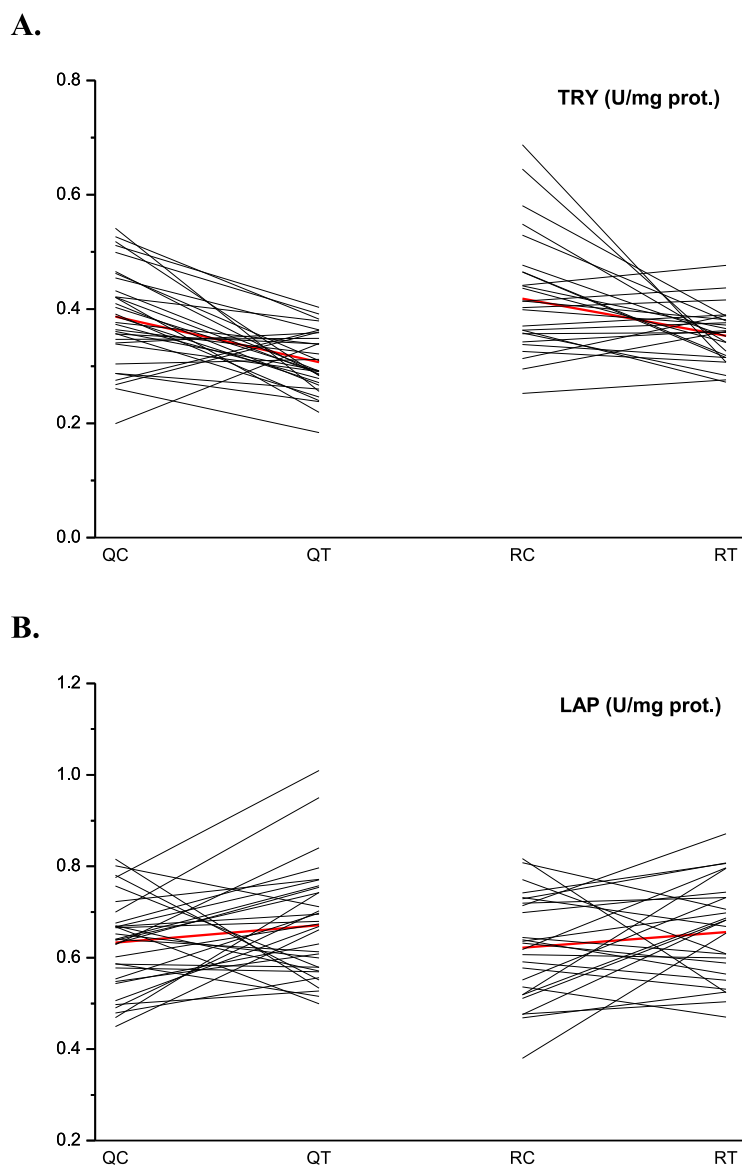
Fig. 1. Norm of reaction plots for the specific activities of: **A** – trypsin (TRY) and, **B** – leucine aminopeptidase (LAP) in 32 families of gypsy moth larvae from the *Quercus* (Q), and 26 families of gypsy moth larvae from the *Robinia* (R) populations, reared on control (C) and tannin-supplemented diets (T).

acid in the diet. In both populations, more families expressed an elevated specific activity of LAP, in the *Quercus* population 21 families and in the *Robinia* population 15 families (Fig. 1B). Two-way ANOVA revealed significant family and "family x treatment" effects for relative midgut mass (RMM) in *Quercus*

larvae, and a significant family effect for RMM in *Robinia* larvae (Table 4). More than half of the total family number in both populations had decreased RMM of larvae reared on the stressful diet, 18 in the *Quercus* and 17 families in the *Robinia* population (Fig. 2).
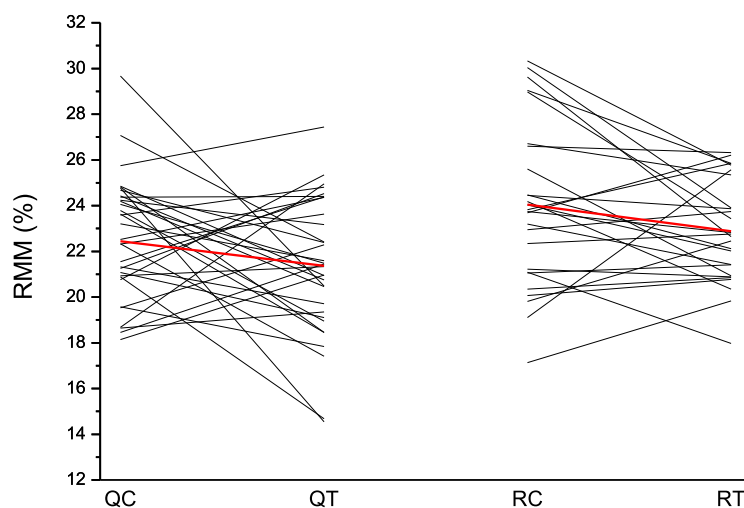
Fig. 2. Norm of reaction plot for relative midgut mass (RMM) in 32 families of gypsy moth larvae from the *Quercus* (Q), and 26 families of gypsy moth larvae from the *Robinia* (R) populations, reared on control (C) and tannin-supplemented diets (T).

Estimation of across-environment (control and stressful environments) genetic correlations revealed positive but non-significant values for the specific activity of trypsin (TRY) in both *Quercus* (r_G=0.180, P>0.05) and *Robinia* (r_G=0.048, P>0.05) populations. The genetic correlations of specific activity of leucine aminopeptidase (LAP) between the two environments were also non-significant within both *Quercus* (r_G =0.198, P>0.05) and *Robinia* (r_G =0.260, P>0.05) populations. A positive significant value of across-environment genetic correlation was recorded for the relative midgut mass (RMM) in the *Robinia* population (r_G=0.50, P<0.01), which was significantly different from "one" (P<0.01).

DISCUSSION

The rearing of gypsy moth larvae from *Quercus* and *Robinia* populations on a tannin-supplemented diet decreased the specific activity of trypsin, while for the specific activity of leucine aminopeptidase, a trend of increase in enzyme activity in response to dietary tannin was noticed (Mrdaković, 2010, submitted, and this study). The protease activities in regulating nutrient balance during the allelochemical stress were associated with leucine aminopeptidase activity, allowing the efficient digestion of oligopeptides and production of more amino acids for uptake. Significant among-population differences were shown for the specific activity of trypsin and relative midgut mass, which, on average, were higher in *Robinia* larvae. In addition, the highest mean value of relative midgut mass, as an indirect measure of food consumption, was detected in *Robinia* larvae reared on the control diet. The higher values of these parameters in larvae from the *Robinia* population indicate adaptive responses to selection during more than 50 generations of feeding on low-protein and tannin-rich locust leaves. The long history of feeding on locust leaves favored larvae that were able to metabolize efficiently stressful food by modulating the activity of some digestive enzymes. High trypsin activity and a high relative consumption rate have been shown as long-term responses to feeding on locust leaves in *Robinia* gypsy moth (Lazarević et al., 2002, 2007). Increased consumption related to nutritionally inadequate food is a compensatory response in herbivorous insects (Slansky and Scriber, 1985; Stockhoff, 1992). Post-ingestive adjustments to nutritional needs can be achieved by a differential release of digestive enzymes (Clissold et al., 2010). In addition to selection

to locust leaf allelochemicals in gypsy moth larvae, nutritionally based maternal effects (Rossiter, 1991) also contribute to expressed differences between the two populations. Between-population variation in the host plant use reveals patterns of local adaptation and suggests the responses to past selection (Ueno et al., 2001a). Although local adaptation has been shown for *Quercus* and *Robinia* populations (Perić Mataruga et al., 1997; Lazarević et al., 2002, 2003; Mrdaković et al., 2013), we did not detect significant "population x treatment" interactions, i.e., analyzed traits reacted in a similar way to dietary tannin regardless of population origin. The process of local adaptation in a population is dependent on the presence of genetic variations in host-use ability (Ueno et al., 2001b). Expression of genetic variations can change as a direct response to stressful environmental conditions (Hoffmann and Merila, 1999). It can be modulated by the influence of phenotypic plasticity on within- and/or among-family variance of a trait. Due to higher selective pressure or higher environmental variance in stressful conditions, traits closely associated with fitness express lower levels of genetic variation compared with morphological and physiological traits (Mousseau and Roff, 1987; Hoffmann and Schiffer, 1998). In both *Quercus* and *Robinia* populations, significant genetic variation of trypsin was detected in the control environment, while genetic variation of leucine aminopeptidase was significant in the stressful environment. Although the use of novel environmental resources can increase the level of genetic variation (Holloway et al, 1990), larger genetic variation of traits was also shown in the commonly encountered environmental conditions (Hawthorne, 1997). The heritability of relative midgut mass of *Robinia* larvae decreased in response to dietary tannin, and was significantly lower than in larvae reared on the control diet. Despite the previously described ongoing adaptation of the *Robinia* population to unfavorable nutrition that can reduce trait heritability (Mrdaković et al., 2011), significant differences in trait heritabilities between the two populations on each of the diets were not detected. Our previous results revealed significant expression of genetic variation of α-glucosidase and lipase activities and their

plasticity within both *Quercus* and *Robinia* populations (Mrdaković et al., 2013), pointing to a potential for the evolution of adaptive plastic responses to stressful environments. The obtained results show the significant variability of trypsin plasticity in larvae from both populations, while the variability of leucine aminopeptidase plasticity was significant in larvae from the *Robinia* population, as is presented by a variation of the norm-of-reaction plots. Plasticity in physiological responses allows adjustment to allelochemically loaded food within a generation. In addition, physiological plasticity is important as a part of the mechanisms of specialization to various host plants. The absence of a significant "family × treatment" effect in the relative midgut mass in larvae from the *Robinia* population could have been due to the pressure of directional selection, i.e. the feeding of previous gypsy moth generations on locust leaves, which reduced the variation in trait plasticity. Genetic variation for plasticity in populations comprises that it can evolve through response to selective pressures of new/stressful environmental conditions. The evolution of optimal phenotypic plasticity could be constrained by strong positive across-environment genetic correlations of traits (Falconer, 1952; Via and Lande, 1985). However, the observed genetic correlations between control and stressful environments for trypsin and leucine aminopeptidase activities were non-significant, and together with the positive across-environment genetic correlation for relative midgut mass in the *Robinia* population which was significantly different from "one", does not represent a constraint for the evolution of plasticity of the analyzed traits.

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