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Desperate times call for desperate measures: short-term use of the common ash tree by gypsy moth larvae (Lepidoptera: Erebidae) under density and starvation stress

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Abstract: Gypsy moth, *Lymantria dispar* L. (Lepidoptera: Erebidae) feeds on a large number of tree species, while ash, *Fraxinus* spp. (Lamiales: Oleaceae) species are considered resistant and are only sporadically eaten. To assess the conditions under which late instar gypsy moth larvae (GML) can temporarily use non-host common ash (CA) (*F. excelsior* L.), and to evaluate their ability to recover from ingestion of this toxic food, we determined the relative growth rate, the relative consumption rate and the amount of produced feces in different laboratory feeding trials. Our report is the first to show that under specific circumstances, the resources acquired after short-term consumption of CA leaves can be utilized for larval growth. We varied the intensity of density and starvation stress prior to feeding on CA leaves. We observed that after moderate stress a group of GML was temporarily capable of coping with CA leaves. Although observed growth and consumption were much lower on CA than on the optimal host oak, *Quercus cerris* L. (Fagales: Fagaceae), CA-oak-switched larvae showed the ability to recover from short-term use of a toxic non-host foliage. This suggests that feeding on CA might enable GML to survive under conditions of food shortage.

Keywords: *Lymantria dispar*; *Fraxinus excelsior*; non-host use; recovery; *Quercus cerris*

INTRODUCTION

The gypsy moth, *Lymantria dispar* L. (Lepidoptera: Erebidae), is a polyphagous pest that feeds on more than 500 plant species [1,2]. Gypsy moth larvae (GML) prefer oaks (*Quercus* spp.) but can also successfully develop on some alder (*Alnus* spp.), birch (*Betula* spp.), larch (*Larix* spp.), poplar (*Populus* spp.), cherry (*Prunus* spp.) and willow (*Salix* spp.) species. However, during outbreaks, they can feed on a wider range of hosts and even incorporate into their diet plants that are usually avoided [3,4].

GML select suitable hosts after dispersal from the hatching site. Early larval instars are dispersed passively through the forest by wind [5], while the 4th and later larval instars can be dispersed by active movement [6]. The movement of later instars is important in the culmination phase of an insect outbreak, when the moth population has exploited all of the resources in one habitat and starts foraging for new food sources. During this quest, GML can travel several kilometers and can experience long periods without food [7]. A less suitable rearing diet and a longer food-deprivation time promote farther movement of late instar larvae [8]. This behavior enables the larvae to escape intraspecific competition caused by overpopulation and diminished host leaf quality after defoliation [9,10], which would otherwise lead to cannibalism [11].

We report the feeding of GML on non-host common ash (CA), *Fraxinus excelsior* L. (Lamiales: Oleaceae) leaves in the wild during the most recent outbreak in eastern Serbia, when the population reached a culmination phase in 2013 (more than 30000 egg

masses per hectare). After completely defoliating all oak trees, the later instar larvae defoliated a neighboring Austrian pine (*Pinus nigra* Arnold) plantation and moved to a beech (*Fagus sylvatica* L.) forest, which was completely defoliated within a few days. Nearby, we found a group of walnut (*Juglans regia* L.) and CA trees infested by 5th and 6th instar GML that were feeding on the leaves. Ash species (*Fraxinus* spp.) are considered resistant hosts because gypsy moth cannot complete development if larvae continuously feed on their leaves [12]. Although some studies have reported ash to be 'sporadically eaten' (reviewed in [2,13,14]) GML never caused visible canopy defoliation. Rejection of ash as a food plant is due to repellency induced by volatile deterrents [15] and feeding inhibition caused by harmful allelochemicals [16].

Our study aimed to explore the conditions under which GML can temporarily use CA leaves in their diet. Preliminary data revealed a complete rejection of CA at low GML densities and short-term starvation that is typical of the latency phase. In this study, we first manipulated the population density and starvation time to simulate the natural environment during the terminal phase of an outbreak. For density/starvation conditions that promote acceptance and short-term feeding on non-host ash leaves, we explored larval ability to recover from the toxic stress, i.e. to continue development on favorable host oak (*Q. cerris* L., Fagales: Fagaceae).

MATERIALS AND METHODS

Insect rearing

To determine conditions under which GML accept ash leaves, we used a standard New Jersey line obtained from the Center for Plant Health Science and Technology (USDA APHIS PPQS&T, MA, USA). For evaluating the ability of GML to recover from short-term feeding on ash leaves, we used a Serbian population from the Varadin-Županja locality (44°57'28.14"N, 19°15'16.64"E). The first experiment was performed in 2017 and the second in 2019. In both experiments, eggs from the middle part of 10 egg masses were cleaned of hairs, mixed, disinfected, transferred to Petri dishes and placed in a climate chamber to initiate hatching. From hatching in May until the 4th instar stage, the larvae were reared at a density of five individuals per Petri dish (120×15 mm) and fed *ad libitum* on an artificial GM diet (MP Biomedicals, LLC, Santa Ana, CA, USA) under controlled conditions as described in [17].

Experimental groups

The consequences of the last gypsy moth outbreak in Serbia for different tree species are presented in Supplementary Fig. S1A (defoliation of oaks) and Fig. S1C, D (defoliation of Austrian pine), Fig. S1B (defoliation of beech). Also, the use of marginal and non-host plants has been recorded (walnut – Fig. S1E, F; ash – Fig. S2A, B). During insect outbreaks, larval densities are within the range of 5000-20000 [18-20] and can sometimes reach up to 50000 larvae per tree [21]. The high larval density in the Serbian GM population is presented in Supplementary Figs. S1E, F and S2A. The density of 10 larvae per liter (larvae/L) in laboratory assays mimics the population density of about 10000 GML per mature tree in the wild, as described by Pavlushin et al. [20]. We also tested the influence of densities of 15 and 20 larvae/L, which corresponded to 15000 and 20000 larvae/L.

In the first experiment, newly molted 4th instar larvae were starved for 3 days in Petri dishes and transferred to glass cylinders (1 L) at densities of 10, 15 and 20 larvae/L (assigned as D10, D15 and D20). These larvae fed on Turkey oak (*Q. cerris*) leaves for 2 days. For each density, 4 glass cylinders were used and 2-3 larvae per cylinder were randomly selected to be exposed to the two starvation treatments (5 or 8 days of starvation). Since at each density larvae were given similar amounts of oak leaves (cca. 10 oak leaves about 10 cm in length), D15 and D20 larvae were food-limited and did not survive an 8-day starvation period. Groups starved for 5 and 8 days at density D10 were assigned as D10-S5 and D10-S8. After density/starvation, the treatment larvae were transferred to Petri dishes and fed individually on CA leaves for 3 days.

The second experiment was performed according to the procedure for the D10-S5 group described above. After 2 days of feeding on oak at a density of 10 larvae/L, the larvae were starved for 5 days and then transferred individually to Petri dishes where they were exposed to three different feeding regimes.

Groups of larvae fed on oak or ash leaves for 3 days were assigned as "Oak" and "Ash", respectively. After 3 days of feeding on ash, the Ash larvae were transferred to oak leaves to monitor the recovery of growth and consumption for 3 days. This experimental group was assigned as "Ash-Oak".

Growth and consumption indices

The leaves and larvae were weighed at the beginning and end of the 3-day feeding trial. The total amount of produced feces was also weighed. The relative growth rate (RGR) and the relative consumption rate (RCR) were calculated on a fresh weight basis for all experimental groups [22,23]. Eight larvae of the D10-S5 in the first experiment and 10 larvae of other experimental groups were analyzed as follows:

$$\text{RGR (relative growth rate)}=(m_{fin}-m_{in})/(t_3\times m_{in});$$

$$\text{RCR (relative consumption rate)}=m_c/(t_3\times m_{in}),$$

where t_3 is the duration of the experiment (3 days), m_{in} is the larval weight at the beginning of the experiment, m_{fin} is the larval weight at the end of the experiment and m_c is the weight of the food consumed.

Statistical analysis

In the first experiment, the normal distribution of RCR and RGR was recorded only in D10-S5 larvae (Shapiro-Wilk's test). As variances were highly nonhomogeneous (Levene's test), we applied Welch ANOVA and the Games-Howell *post hoc* test [24] to estimate significant differences among ash larvae from the different density/starvation experimental groups. Since in the second experiment the $\sqrt{x+0.5}$-transformed values of RGR and square root-transformed values of RCR satisfied the assumptions of normality and homoscedacity, we tested the significance of the Oak vs. Ash and Oak vs. Ash-Oak differences by the t-test for independent samples, and the Ash vs. Ash-Oak difference by the t-test for dependent samples. The data on the amount of produced feces in the group fed on ash did not have a normal distribution. The Oak vs. Ash-Oak comparison was performed using the t-test for independent samples; Oak vs. Ash was performed using the Kruskal-Walis test and Ash vs. Ash-Oak by the Wilcoxon matched paired test.

RESULTS

Density and starvation effects on GML growth and consumption on ash leaves

We showed that GML can feed, grow and produce feces on common ash leaves under specific circumstances when the density/starvation stress was not too strong (Figs. 1 and 2). Six out of 8 D10-S5 GML started to feed on CA leaves and gained ~27% of their initial body weight. It can be seen in Fig. 1 that the population density preceding feeding on CA significantly affected all examined traits (RGR:$F_{2, 13.82}$=6.99, P=0.0080; RCR: $F_{1, 7.01}$=9.21, P=0.0189; feces: $F_{1, 9.03}$=5.82, P=0.0390). Our results show that densities above 10 larvae/L followed by 5 days of starvation disabled the larvae to cope with non-host food. Namely, none of the D15 and D20 larvae gained weight. We showed that a prolonged starvation time significantly lowered the RGR ($F_{1, 8.31}$=11.94, P=0.0082) and feces production ($F_{1, 7.27}$=6.95, P=0.0326) while the reduction of RCR was marginally significant ($F_{1, 8.65}$=3.46, P=0.0972) (Fig. 2, Supplementary Fig. S2C, D, E and F). Leaf consumption was recorded in 7 out of 10 D10-S8 larvae, but only 2 of them gained weight. On average, they lost about 8% of their weight.

Growth and consumption recovery after feeding on ash

Results from assessing the ability of GML to recover from short-term toxic stress are presented in Fig. 3. In comparison to the optimal host Turkey oak, GML significantly reduced growth (t=15.67, P<0.0001), consumption (t=11.57, P<0.0001) and feces production (H=14.29, P=0.0002) on CA. After switching from CA to oak leaves, all indices were significantly increased (RGR: t=9.91, P<0.0001; RCR: t=11.04, P<0.0001; feces: Z=2.80, P=0.0051). After the switch, the RGR did not reach the RGR value on oak (t=2.51, P=0.0220), while consumption and feces production completely recovered and did not differ from the Oak group (RCR: t=0.91, P=0.3726; feces: H=0.09, P=0.7620).

DISCUSSION

Our laboratory results confirmed the field observations of GML feeding on CA leaves. However, growth, consumption and feces production in D10-S5 ash lar-

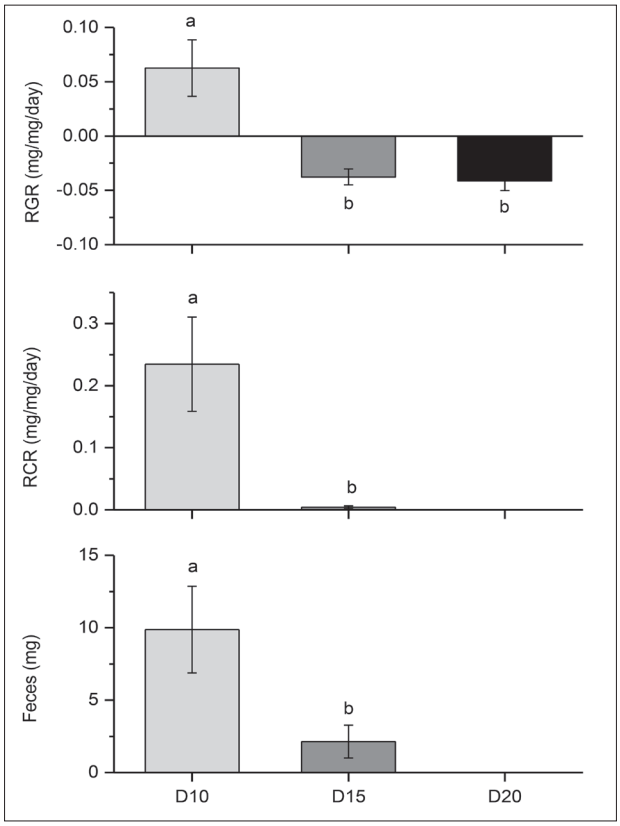

Fig. 1. Effects of population density on the relative growth rate (RGR), the relative consumption rate (RCR) and feces production in gypsy moth larvae starved for 5 days before feeding on common ash leaves. Densities of 10, 15 and 20 larvae/L were assigned as D10, D15 and D20, respectively. Bars marked by letters a and b are significantly different (P<0.05).

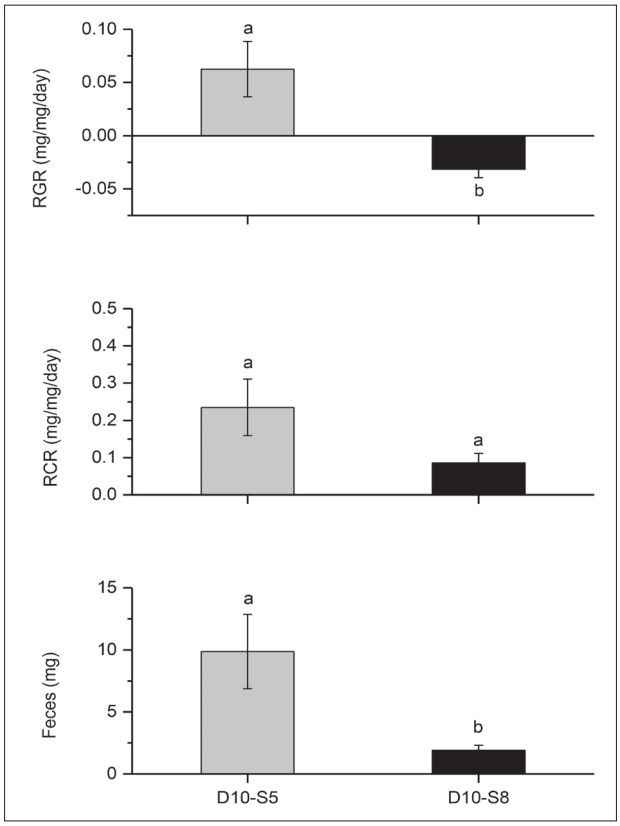

Fig. 2. Effects of starvation duration on the relative growth rate (RGR), the relative consumption rate (RCR) and feces production in gypsy moth larvae at a population density of 10 larvae/L that fed on common ash leaves after 5 (D10-S5) or 8 (D10-S8) days of starvation. Significant differences are presented by letters a and b displayed above bars (P<0.05).

vae were clearly below the values obtained on an optimal host after the same density/starvation stress. Data from other authors on larvae reared under optimal conditions (suitable hosts, low density, no starvation) have also shown much higher values of nutritional indices [17,25-28].

CA and other *Fraxinus* species are largely immune to attacks by GML because the leaves contain many deterrents, toxins and digestive inhibitors [29]. Besides, compared to suitable oaks, CA is associated with a lower number of other insect pests, indicating the strong antiherbivory effects of its compounds [30]. Extensive work on the relationship between green ash (GA), *F. pennsylvanica* Marsh. and GML revealed feeding inhibitory, repellent, toxic and growth-reducing effects of leaf ethyl acetate extracts, which, as the authors suggested, contained glucosides, phenolics and terpenoids [15,16,31]. Feeding on GA leaves, starting from any of the 1st to 4th instars, disabled pupation in GM [12]. A more than 40-times lower extract concentration in the diet as compared to fresh leaves was sufficient to provoke such a response [31]. Feeding inhibitory and growth-reducing effects were also recorded in our experiment. These effects are expected to be expressed more for CA than GA leaves as they emit more of the deterrent volatile α-farnesene [15,32] and contain more diverse, potentially toxic and/or antinutritive coumarins, flavonoids and secoiridoids [33-36].

The reduced RGR on CA could be a result both of behavioral (low RCR) and physiological effects (high cost of food processing). Iridoid glucosides are known for their bitter taste and they thus have both deterrent and growth-reducing effects on GML [37]. The secoiridoid oleuropein that is characteristic of *Fraxinus* species, including CA [34,35], after the activation by β-glucosidase acts as a protein denaturant, which

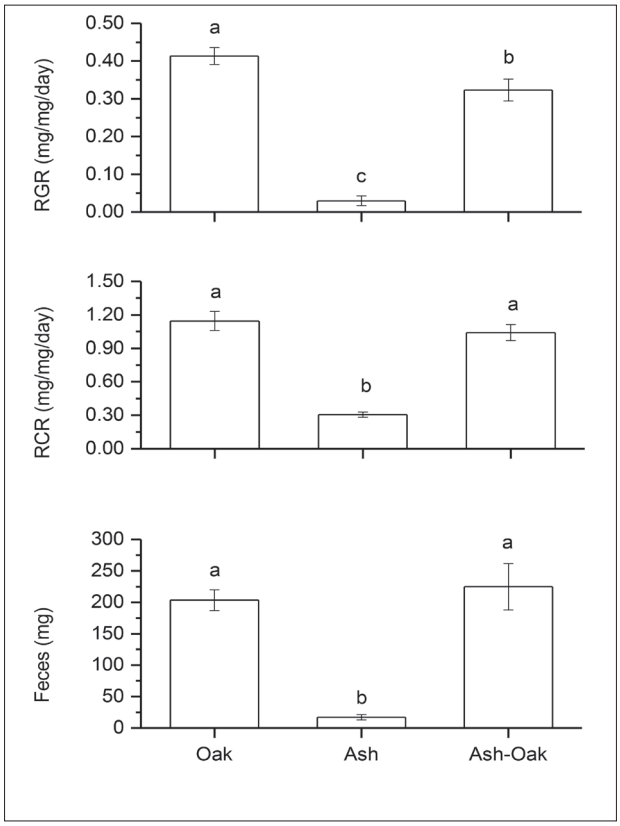

Fig. 3. Relative growth rate (RGR), relative consumption rate (RCR) and feces production in larvae fed on oak and ash, and larvae that switched from ash to oak. Before the feeding trials, larvae were maintained at a density of 10 larvae/L and were then exposed to 5 days of starvation. Significantly different trait values are marked by letters a, b, c ($P<0.05$).

lowers the nutritive value of dietary proteins [38]. CA leaves contain glucosides of the coumarins fraxetin and esculetin as well as glucosides of the flavonoids quercetin and kaempferol [33] that can act as prooxidants and enzyme inhibitors. For example, quercetin is known as a prooxidant and a strong trypsin inhibitor [39,40]. To cope with dietary protein-crosslinking, the generation of free radicals and impairment of protein digestion, insects activate various physiological mechanisms such as glycine secretion [41], induction of antioxidative defense and detoxification [39], as well as changes in the levels and isozyme patterns of digestive enzymes [42]. Since these processes are energetically costly, to cope with CA leaf chemistry GML should divert part of the assimilated food from growth towards energy metabolism.

Insects have evolved various adaptive responses against plant chemical defenses at the level of feeding behavior, physiology and metabolism [43]. The efficacy of these responses depends on abiotic and biotic contexts [44]. As a species with eruptive population dynamics, GM encounters fluctuations in population density that affect interactions with host and non-host plants. Although GM maintains a high level of polyphagy in different phases of population growth, it was reported that the ecological preference for non-host ash species and several unsuitable hosts is increased during the culmination phase [3]. Densities above 10 larvae/L in our laboratory assays involved food limitation and increased physical contact among larvae, which might further impair feeding, increase larval activity and, together with food limitation, lead to the depletion of energy stores. For instance, changes in the activity of medial neurosecretory neurons in GML suggest intensive carbohydrate metabolism in response to high-density stress [45]. Reduced energy stores might affect resistance to starvation and the ability to detoxify or eliminate harmful allelochemicals from ash leaves, and thus may explain the inability of the larvae to gain weight after experiencing high-density or prolonged starvation.

Similarly, starvation also provokes hormonal and metabolic reorganization in insects [46,47]. Depletion of lipids and carbohydrates and a decline in metabolic rate have been recorded in GM after food removal [48]. It is likely that the 8 days of starvation in our experiment reduced energy stores that could be allocated towards detoxification. Likewise, a significant synergistic interaction between density-dependent starvation and toxin concentration for adult eclosion in *Drosophila melanogaster* Meigen has been recorded [49], and it was suggested that a "low food supply may reduce (the) metabolic rate of the flies and thus reduce detoxification rates". Our preliminary data with starvation duration below 5 days revealed that GML were not willing to feed on non-host CA, regardless of larval density. Accordingly, larvae that are not sufficiently starved are too choosy, while larvae that have been starved for longer periods are too exhausted to temporarily use CA leaves.

The composite generalist feeding habit of the gypsy moth mirrored in significant variations in fitness, behavior and activities of detoxification enzymes [50-52] might facilitate the temporary use of non-host ash leaves. After about a 90% reduction of RGR and a 75% reduction of RCR in Ash larvae, only 3 days on oak

leaves were sufficient to reach RGR and RCR values that were only 20% and 10%, respectively, lower than in oak larvae. We suggest that despite the metabolic costs of processing inadequate food, individuals capable of metabolizing toxic and antinutritive compounds might avoid starvation and thus have an advantage under extreme conditions. Additionally, our results on the repellent and growth-reducing effects of CA point to a possible application of its leaf extracts as a natural eco-friendly biopesticide for GML management.

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Author contributions: SM conceived and designed the study. MP and JD conducted all of the experiments. IK statistically analyzed the data. SM and JL wrote the manuscript with assistance from IK, MP, and JD. The final manuscript was read and approved by all authors.

Conflicts of interest disclosure: There are no conflicts of interest to declare.

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Supplementary Material

The Supplementary Material is available at: http://serbiosoc.org.rs/NewUploads/Uploads/Milanovic%20et%20al_4780_Supplementary%20Material.pdf