Foliage maturity of *Quercus ilex* affects the larval development of a Croatian coastal population of *Lymantria dispar* (Lepidoptera: Erebidae)

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Abstract: Gypsy moth (*Lymantria dispar*) is one of the most important forest pests in the world. Numerous previous studies focused only on different host tree species, but small number of them on foliage age. Since recent genetic analyses showed that there are significant differences between Croatian continental and coastal population we investigated coastal population since there was no previous research. For this research juvenile and mature foliage of Holm oak (*Quercus ilex*) was used. Larval development was investigated in two laboratory experiments. One experiment involved rearing trials consisting of 50 individual larvae per treatment while a parallel experiment investigated gregarious feeding conditions using 120 larvae in a rearing treatment. Larval mortality was lower and development time shorter for individuals reared on juvenile foliage. High pupation success in both the individual and group rearing experiment for larvae reared on juvenile foliage was also observed. To conclude, our results showed high mortality, poor larval development and low pupation success in larvae reared on mature foliage. This research is significant because in the aspect of ongoing climate changes there is a possibility that gypsy moth will move to the north and shift its distribution by expanding into new climatic area.

Keywords: Gypsy moth, Holm oak, defoliation, outbreak, host plant

1 Introduction

The gypsy moth (*Lymantria dispar* L., Lepidoptera, Erebidae) is a widely known defoliating forest pest with periodic outbreaks [1,2]. The distribution of this species is primarily within Europe, Asia and North Africa, but in 1868 it was also introduced to North America [3,4]. Defoliation by gypsy moths, especially in consecutive years, not only weakens trees by making them increasingly vulnerable to secondary pests (such as jewel (Buprestidae) and longhorned (Cerambycidae) beetles) and fungal infections [5-8], but also disturbs the natural ecological processes within forest environments due to increased amounts of frass, leaf particles and sunlight [9,10]. Preceded by a winter diapause in the egg chorion, gypsy moth larvae hatch in early spring, before moving up the plant stem in search of suitable food where they are often also dispersed by the wind (“ballooning”) [9,11-13]. Gypsy moth larvae are highly polyphagous and feed on more than 300 plant species, however *Quercus* sp. L. oaks are preferred [4,12,14].

The distribution of the gypsy moth in Croatia spans the continental and coastal region separated by the Dinaric Alps, however recent genetic analyses showed significant differentiation between the populations occupying these two regions [15]. There are clear differences in the population dynamics between the continental and coastal populations. Outbreaks in the continental region occur every 10 to 11 years [2] and are spatially synchronised throughout Central Europe (“Moran effect”), while the coastal populations have more frequent outbreaks and are not synchronised like those in the continental region.
Gypsy moth response to holm oak leaf age

2 Material and Methods

Gypsy moth egg masses were collected on the island of Cres, Croatia (Punta Križa; 44° 38’ 12” N, 14° 29’ 30” E) in the autumn of 2011. The dominant host plant at this locality is the holm oak. The egg masses were kept in petri dishes at 4 °C until the start of the experiment (25 April 2012), when the temperature was then increased to 20°C. The first larvae hatched on 2 May 2012, 7 days after temperature increase. Throughout the experiment, foliage of holm oaks was collected from forest stands on the island of Krk (Čižići; 45° 09’ 03” N, 14° 35’ 49” E), and a fresh stack of foliage was delivered every week to the laboratory. To maintain freshness, the collected branches were placed in buckets filled with water and kept in a cold room. Throughout the feeding experiment, gypsy moth larvae were kept at 20±1 °C, a relative humidity of 65 % and a photoperiod of 16h:8h (L:D). After hatching, first to third instar (L1 – L3) gypsy moth larvae were kept in glass cylinders with a mixture of juvenile and mature holm oak foliage.

At the start of Experiment 1, larvae were divided into two sub-experiments. At the end of L3, 100 larvae were placed individually in Petri dishes and labeled. From fourth instar (L4) to pupation, 50 larvae were fed with juvenile foliage and another 50 larvae with mature foliage. The larvae were weighed daily on a Sartorius BD ED 100 (ATL 224-I) analytical laboratory balance (limit 0.0 001 g). The larvae were weighed daily on a Sartorius BD ED 100 (ATL 224-I) analytical laboratory balance (limit 0.0 001 g). The larvae were weighed and sexed. The last larvae pupated at 2 July 2012, however the larvae which never pupated were weighed until the 29 July 2012 when the experiment finished.

Experiment 2 was conducted in two glass cylinders with a total of 240 gypsy moth larvae. With the completion of the L3 instar, 120 larvae were put in the cylinder with only juvenile holm oak foliage and in the second cylinder 120 larvae were provided only mature foliage. The rearing conditions were the same as in Experiment 1. During Experiment 2 however the larvae were not weighed, but larval development was recorded until pupation and larval mortality recorded daily. At the termination of Experiment 2, 20 pupae were randomly selected from both feeding treatments (i.e. juvenile or mature foliage) and were weighed and sexed.

Statistical analysis was conducted with Real Statistics Add-In and with Data Analysis Toolkit, both in Microsoft Excel 2007 (Microsoft® 2007) and with IBM® SPSS® Statistics Version 24 (IBM® 2016). Data were inspected for normality prior to statistical analyses. The two rearing groups were compared using either two-tailed Student’s t-test or Mann-Whitney Test. For the comparison of survival rates between two rearing groups, a Fisher’s test was used. The level of significance was set at α=0.05.
3 Results

At the beginning of Experiment 1 L4 larvae were randomly selected from the bulk of individuals reared on the mixture of juvenile and mature holm oak foliage, where they were observed to be mainly feeding on juvenile foliage. At the start of the experiment, L4 larvae selected for the group to be reared on juvenile foliage weighed on average 0.05±0.01 g (n=50), while the average initial larval mass for those selected for the treatment group reared on mature foliage was 0.07±0.00 g (n=50). A Mann-Whitney Test (U=617, n₁=n₂=49, P < 0.05 two tailed) showed that despite the random allocation, the larvae chosen for the feeding trial with mature holm oak foliage were significantly heavier than those chosen for the feeding trial with juvenile foliage. The duration and mode of development of the larvae throughout L4 and L5 are displayed in Table 1 and Figs 1 and 2. There was a significant difference between the development of gypsy moth larvae in L4 and L5 on the juvenile and mature foliage. Of the 50 larvae used in the individual rearing experiment with juvenile foliage, 23 were males and 26 females (and one larva died during the experiment). In the treatment with larvae reared on the mature foliage, only 3 larvae developed to pupae and all were males: larval mortality in this group was extremely high (Fig 3). The majority of larvae fed with juvenile foliage pupated during the sixth instar (males 42.5%, females 57.5%). Average pupal mass of the insects reared on juvenile foliage was 0.94±0.02 (females) and 0.50±0.01 g (males). While no differences in female pupal mass were observed in individuals that pupated after the fifth and sixth instar, male pupal mass was strongly affected by the instar stage. In these cases, male larvae that pupated after the sixth instar were significantly larger than those that pupated after the fifth instar (Fig 4).

Table 1. Duration (days, means±SE) of instar 4 and instar 5.

<table>
<thead>
<tr>
<th></th>
<th>juvenile foliage</th>
<th>mature foliage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Instar 4</td>
<td>5.7±0.2 aA (n=50)</td>
<td>19.0±1.1 bA (n=50)</td>
</tr>
<tr>
<td>Instar 5</td>
<td>8.7±0.3 aB (n=49)</td>
<td>12.4±1.4 aB (n=28)</td>
</tr>
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</table>

Fig. 1. Increase in mass (g, means±SE) of L. dispar larvae throughout instar 4 feeding on juvenile and mature foliage. The numbers above represent number of larvae in certain days during instar 4.

Fig. 2. Increase in mass (g, means±SE) of L. dispar larvae throughout instar 5 feeding on juvenile and mature foliage. The numbers above represent number of larvae in certain days during instar 5.

Fig. 3. Mortality (%) of larvae feeding on juvenile (n=50) and mature (n=50) foliage of Q. ilex.
Results from Experiment 2 also illustrated high mortality rates for larvae reared on mature foliage (Fig 5) and significant differences in larval development (Fig 6). 98% of larvae on juvenile foliage pupated successfully while only 63% of those reared on mature foliage pupated (Fisher’s exact test, p < 0.05). Pupal mass of females and males randomly selected from Experiment 2 were compared between the treatments of juvenile and mature foliage (Fig 7). For both sexes, larvae fed with juvenile foliage attained pupal masses more than twice that of the pupae from the mature foliage treatment respectively (females 0.99±0.05 and 0.47±0.04 g; males 0.48±0.02 and 0.24±0.02 g). Comparisons of female and male pupal masses from Experiment 1 and Experiment 2 reared on juvenile foliage showed no significant differences [females (Student’s t-test, t=(21)=−0.94; Experiment 1=0.94±0.02 g; Experiment 2=0.99±0.05) and males (Student’s t-test, t=(23)=0.92; Experiment 1=0.50±0.01 g; Experiment 2=0.48±0.02 g)].

4 Discussion

The results illustrated significant differences in time of the development, larval mass, mortality and pupal mass between the rearing treatments. In general, larvae feeding on the preferred juvenile foliage grow larger, have larger pupae and produce more fecund adult females [33]. The coastal population of the gypsy moth on the island of Cres appears to have developed different behavioural patterns that may be influenced by the local climate and host plants.

Previous palatability studies have shown that there are no host preferences of gypsy moth larvae...
among sessile oak, white poplar (*Populus alba* L.) and holm oak (*Quercus ilex* L.) (Schafellner et al., unpublished data). Foliage preference may be related to the histological and physical characteristics of holm oak foliage. During both experiments it was observed that gypsy moth larvae experienced difficulties with the mastication of the mature foliage, which is very hard and leathery and has tough serrated margins [32, 34]. This supports the findings of Scriber and Slansky [35] that illustrated that as the foliage matures its palatability and quality declines. Supporting this further are Acevedo et al. [36], who found that stronger mandibles are needed to eat tougher food. This may explain why during gypsy moth outbreaks even the oldest foliage is devoured as it is probable that the late instar larvae have developed a large enough head capsule and strong mandibles. A widely investigated aspect is to understand the relationships between host plants and herbivorous insects is the level of tannin in the foliage. Tannins are accepted as an important resistance factor against herbivory by insects [38]. According to Makkar et al. [37] the levels of tannin in holm oak foliage increases as the foliage matures. Furthermore, the results of Bourchier and Nealis [38] study using tannin-supplemented diets were especially pronounced in later instars, where females on a unsupplemented control diet grew at twice the rate of larvae on tannin-supplemented diets. They have used 5 groups. Control group with a 0 %, 0.5 %, 1.5 %, 2.5 % and 5 % of tannin. Statistical significant difference was only observed on groups 2.5 % and 5 %.

Another difference between the continental and coastal region is the dominant tree species. In the continental region of Croatia, the preferred food for the gypsy moth is pedunculate oak foliage. As pedunculate oak is one of the most important tree species in Croatia (both ecologically and economically) a great deal of research on its pests, especially defoliators, has already been conducted [40-42]. Connection between the budburst of pedunculate oak and the gypsy moth larvae is important since emergence must be harmonized within a short period between budburst and the point in time when foliage quality is too low [43]. According to Seletković and Tikvić [44], in the continental region pedunculate oak starts to budburst at the beginning of April (the mean value for 1993 was 19 April and for 1994 was 6 April). Whilst the holm oak is an evergreen tree species, it also has a budburst in spring. According to much research that has covered a wide area of the Mediterranean Basin, holm oak budburst occurs in the period from the end of March to the beginning of June, depending on the location [45-49]. In Croatia the budburst of holm oak occurs at the beginning of May (Lukić, personal observation). Considering this dissimilarity in the budburst of these two oak species, an important question which remains to be investigated is when do the gypsy moth larvae emerge in the coastal region of Croatia.

Our results revealed high mortality and poor development of gypsy moth larvae when reared on mature foliage of the holm oak. This is likely influenced by many factors and further comparative research should be performed on leaf chemistry and nutrition value in order to better understand the causes and mechanisms behind this result. Additionally, it remains to be resolved whether the emergence of gypsy moth larvae coincides with the budburst of holm oak and, what is the food preference of neonate larvae if the two phenomena are not synchronised.

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